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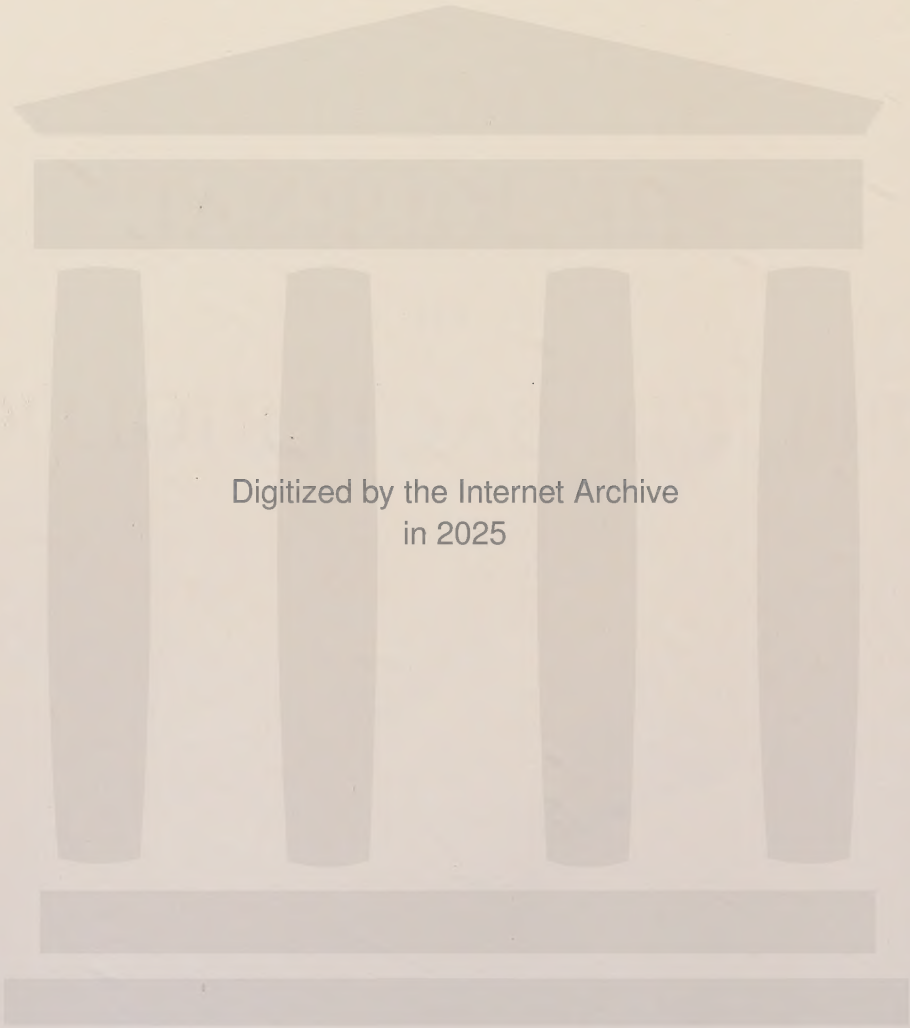
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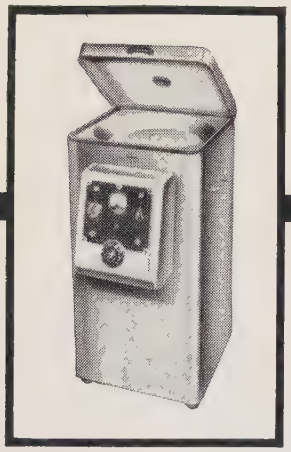

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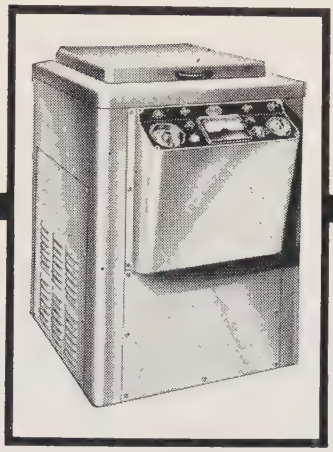

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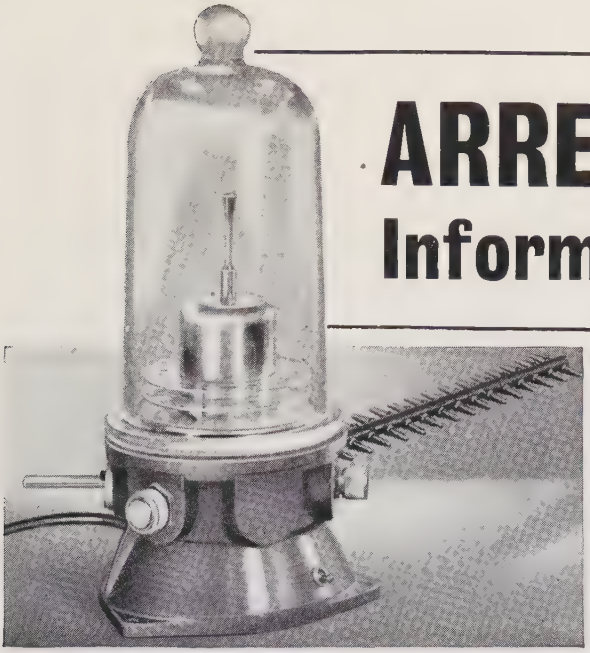

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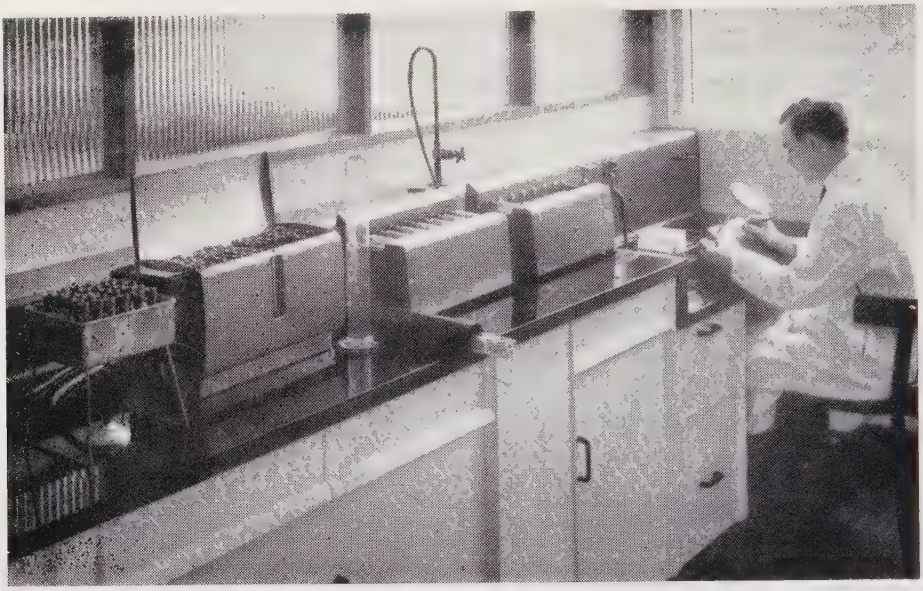

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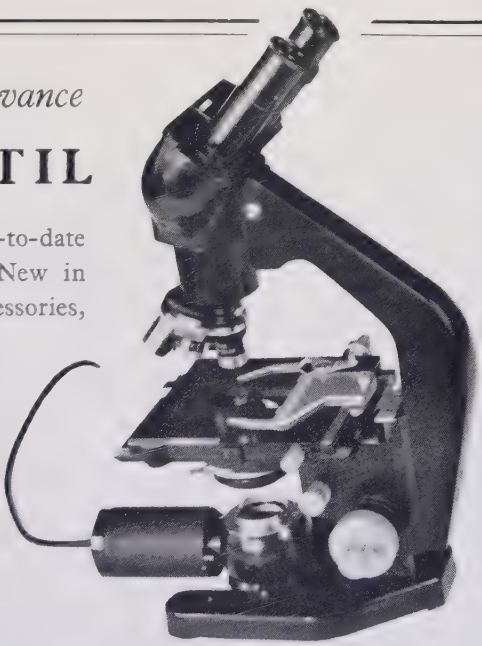

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Reviews of the Progress of Dairy Science :

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No. 1, Vol. 21 of the Journal of Applied Bacteriology was issued on 12 August, 1958.

Taysum, D. H. (1958). *J. appl. Bact.* **21** (2), 161–173.

THE NUMBERS AND GROWTH RATES OF THE BACTERIA IN *HEVEA* LATEX, AMMONIATED FIELD LATEX AND AMMONIATED LATEX CONCENTRATE

By D. H. TAYSUM

The Rubber Research Institute of Malaya, Kuala Lumpur, Malaya

SUMMARY: The rates of growth of bacteria on *Hevea* latex systems in the presence and absence of ammonia have been derived from colony count data obtained on a modified Kligler's iron agar medium. For fresh latex, ammoniated field latex, and ammoniated latex concentrate, variations in the bacterial populations encountered are given for a period of about one year's testing, with some data on commercial samples of latex concentrate. Field latex at routine collection contained about 8×10^6 bacteria/ml. This could be reduced by 62% by the use of sterile tapping cups. In the routine latex, the mixed population grew logarithmically after a lag phase of 2½–3 hr. In clean latex the lag phase was extended to 4 hr. On ammoniation to 0·3% (w/w), the population was reduced over about 2 hr, but subsequently logarithmic growth recurred, without a lag phase. On concentration and further ammoniation to 0·7% (w/w), the count dropped still further, levels of 10/ml or less being achieved in clean conditions, but very slow logarithmic growth again occurred with a lag phase preceding it.

A modification of Kliger's iron agar medium for enumerating the bacteria in *Hevea* latex systems, both ammoniated and unammoniated, described by Taysum (1956), has now been used extensively to follow the development of these organisms in latices from routine production, and the results are described below. From them, estimates have been derived of the mean generation times of the mixed populations found therein.

Materials and Methods

Latices. Three latex systems were examined. (i) Fresh latex, tapped either under normal (dirty) conditions or with the use of cleaned or sterile chutes and cups. The mean dry rubber content of these latices was 36%. (ii) Ammoniated field latex, obtained from normally collected fresh latex not more than 6½ hr old, by adding about 0·3% (w/w) of gaseous ammonia (on the whole latex). Average dry rubber contents, concentrations of ammonia on the water phase, and sources, are indicated for the individual samples. (iii) Ammoniated latex concentrate, obtained by centrifuging ammoniated field latex and ammoniating further, directly afterwards.

Sources of field latex. All field latices used in the evaluation of the bacterial contamination of fresh latex, ammoniated field latex and ammoniated latex concentrate were derived from areas of unselected seedling trees. In these areas, trees had been raised directly from seed of unknown origin without subjecting them to bud grafting. The resulting 'seedling' trees were 26 years old at the height of the experiment. In the case of commercial samples of ammoniated latex concentrate, the source of

parent field latex was entirely mixed, the latter being derived from areas of mixed clonal seedlings, unselected seedlings and a variety of clonal buddings.

Tapping and collecting procedure. For the experimental latices and their derivatives the tapping was on a half-spiral cut and was performed on alternate days as routine. On tapping days the 'scrap rubber' on the cuts was removed by the tapper and a fresh cross-section of bark and latex vessels taken by his knife. Tapping started at about 6.45 a.m. and proceeded to about 9.30 a.m. in these areas. For computation of growth rates and for the analysis of inocula for fresh latex, the tapping was performed as a separate procedure by a skilled tapper; again alternate daily operation on a half-spiral cut was established.

Latex normally runs from the half-spiral cut, down a short vertical channel, on to a small steel chute and into a ceramic cup. The tapper collected latex from the cups at 11–11.30 a.m. and brought it to the laboratories in twin 4 gal buckets, a distance of some 300 yards, to be ammoniated and centrifuged as required.

Experiments performed to reveal the sources of bacterial infection consisted in substituting sterile or well cleaned cups for normally treated ones, using sterile tapping knives and introducing sterile or well cleaned chutes. In all, some 80 unselected seedlings were analyzed in this way. The cups were well cleaned with dilute detergent solution and washed with water. After adding 5 ml of sterile isotonic saline and covering with a brown paper hood, they were sterilized by autoclaving at 20 lb/in.^{2} for 20 min. The saline was poured through a small opening in the paper just before the first latex entered the cup.

Sampling. In assessing the various contributions to infection, latex was sampled from individual cups, after allowing it to collect there for 30–45 min from the start of tapping, with a sterile pipette. This time permitted an approximate equilibrium of infection to be established. Before this, the comparatively small volumes of latex were grossly contaminated from the surfaces with which they came into contact. As the volume of latex in the cup increased, the amount of infection fell to a level which was approximately constant until the lag phase was complete.

With systems in which coagulation had already occurred sampling was from the 'serum' phase, after stirring. Counts derived in this way were probably low since some bacteria must have been embedded in the coagula.

Colony counts. The plating method was used, with 1 ml inocula from serial tenfold dilutions, the medium being the modified Kligler's iron agar of Taysum (1956). Deep plates were used throughout (14 ml/dish). Incubation of parallel plates under hydrogen, nitrogen, carbon dioxide and mixtures of these, did not increase the viable count; in fact a decrease beyond that expected from the loss of obligate aerobes alone was observed. There seemed therefore no need to have special tests to count obligate anaerobes, which are assumed to be included in the specific value obtained.

In general, for field latices, five plates of the dilution expected to give 40–80 colonies/plate (say, the 10^{-5} dilution) were set up, together with two plates from each of the next higher (10^{-6}) and lower (10^{-4}) dilutions. This procedure, though tedious, virtually eliminated experimental failures. The plates were incubated at 30° for 2 days. A similar procedure was adopted for concentrates, except that the inocula additionally consisted of 5, 2 and 1 ml and incubation was for 5–6 days.

Results

Fresh latex

Bacterial content in the field. Counts made on 80 samples taken from normal tapping cups gave a mean value of $8{\cdot}02 \pm 0{\cdot}06 \times 10^6$ bacteria/ml for the initial population. It should be emphasized that this count applied to an area where precoagulation was rare; more recent experiments suggest that where precoagulation is manifest the populations may be considerably higher.

The effect of clean or sterile cups and chutes. In the following analysis of the contribution to the flora of fresh latex made by the tapping cup, tapping chute and tapping panel the percentages are all based upon the average initial population for the normal conditions. No result can be given a greater accuracy than $\pm 1\%$. Eighty separate measurements contributed to the results for each improvement in technique. The average cup contribution proved to be about 62% of the total bacteria present; i.e. replacing the cup with a similar but sterile vessel reduced the count by 62%. The use of cups cleaned and washed with the anionic detergent Teepol (0·1% v/v aqueous solution) but not finally sterilized, reduced the population by 56%. A larger cup contribution might have been expected, but in fact the tapping chute contributed 20%. The substitution of a Teepol-washed but not sterilized chute gave a 15% reduction. The deduced contribution of the tapping panel was thus 18% of the initial colony count. That this was so high was surprising, in view of the fact that the 'scrap rubber' was removed by the tapper and a fresh cross-section of bark and latex vessels taken by his knife; but examination of the 'scrap rubber' revealed that its outer surface carried a mass of bacterial colonies, often brightly coloured, and evidently the majority of the bacteria infecting the panel came from this source, the transferring vectors being the tapper's knife and hands. The substitution of a sterile tapping knife gave no significant reduction in colony count, the maximum observed reduction being 2%. It seems therefore that the tapping knife behaved more as a vector for transfer rather than as a significant source of infection.

Bacterial content on arrival at the factory or laboratory. Counts on 12 samples of bulk latex arriving at the factory gave a mean bacterial content of $4{\cdot}2 \pm 0{\cdot}38 \times 10^7$ bacteria/ml, for the area for which a mean cup value has already been presented, at least 5 hr having elapsed since tapping began. This figure would obviously be subject to greater random fluctuations than the corresponding cup value as infection from the non-sterile buckets, though probably small, was uncontrolled and the precise time taken in carrying latex to the factory varied somewhat from day to day.

It is not in general possible to perform an accurate factory evaluation for a whole estate of, say, 3,000 acres, and to derive data of bacteriological value thereby; lengths of journey from field to factory varied widely and methods of collection are often quite different for the more remote areas. In part to overcome this, 53 determinations were made in a period of about 1 year on samples taken once a week from bulk latex from a block of 26 year old seedlings in an experiment in which control of procedure was a little more rigorous than usual. Tapping was done on alternate days. It started at 6.30 a.m. and the latex was collected at 10.30 a.m., while counting was performed at 3 p.m., this delay serving to widen the differences between the counts. Apart

from the delayed counting, these samples closely reflected the best aspects of normal procedure. The mean dry rubber content was 36%. The mean count was $1{\cdot}93\times10^8$ bacteria/ml, with upper and lower limits of $3{\cdot}0\times10^9$ and $4{\cdot}4\times10^6$/ml. Actually, many of the larger deviations from the mean could be directly attributed to local conditions, principally heavy previous rainfall; this invariably increased the count. The delay to 3 p.m. should be noted in interpreting the lethal effect of ammonia.

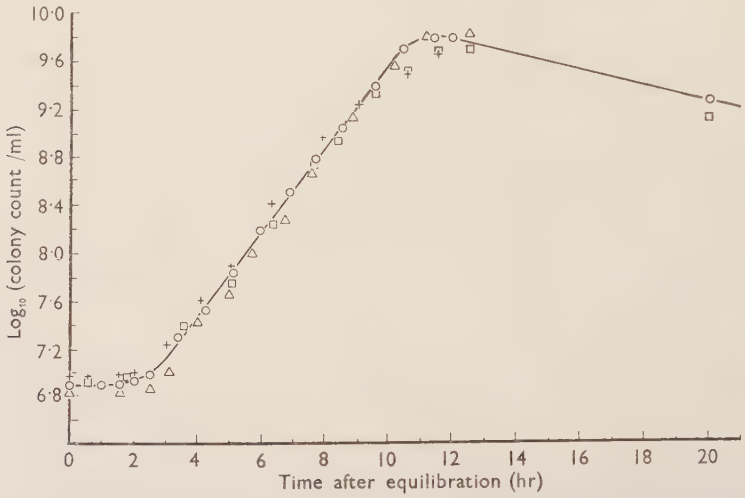

Fig. 1. The growth of the bacterial flora in four samples of fresh *Hevea* latex, collected under routine estate conditions but taken from separate cups.

The course of development of the mixed population. To establish the course of this multiplication, the changes in numbers with time (at air temperature, *c.* 28°) were followed in four latices taken from cups in routine estate use, i.e. with no pretence to hygiene. The results are shown in Fig. 1. As stated previously, an equilibrium time of 30–45 min was allowed. Clearly, the changes correspond to normal growth curves of pure cultures, with lag and logarithmic phases. The lag phase occupied $2\frac{1}{2}$–3 hr. The mean generation time derived from the logarithmic phase was slightly less than 1 hr, but in fact the slopes of the graphs representing the four separate logarithmic phases are statistically different. The most accurate single determination of the mean generation time was 52 min. The duration of the logarithmic phase was 7–10 hr, with a mean value of 8 hr. Latex begins to thicken visibly when the population approaches 10^9/ml; after that, results become increasingly difficult to obtain and after natural coagulation only broad statements concerning numbers can be made. On coagulation the colony count is usually about 4×10^9/ml. In these experiments the population increased slightly beyond the level at coagulation, to reach a maximum of $6{\cdot}8\times10^9$/ml. During further storage there was a rapid decline and in the present case the count fell to 3×10^7/ml after 48 hr. A corresponding series of experiments with 8 samples of latex from sterile cups gave curves of similar form, the only striking difference being in the duration of the lag phase, which for these cleaner conditions ranged from $3\frac{1}{2}$–$4\frac{3}{4}$ hr, with a mean of just over 4 hr. Thus

it seems that the lag phase was markedly longer for the more hygienic conditions; a possible application of this is presented in the Discussion.

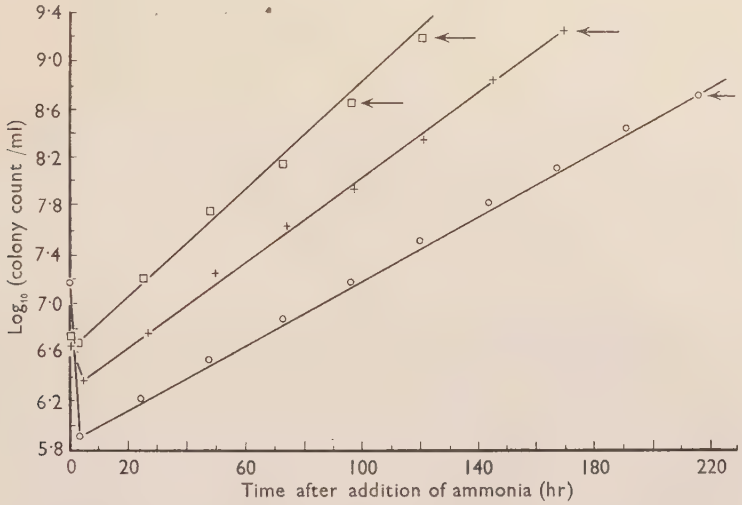

Fig. 2. The immediate lethal effect of ammoniation to different levels on the bacterial flora in three samples of fresh *Hevea* latex and the subsequent growth of the survivors.
Ammonia concentration (% w/w on the water phase): circles, 0·49; crosses, 0·46; squares, 0·43. The arrows indicate the times at which visible thickening of the latex was observed.

Ammoniated field latex

Ammonia was added to eight of the samples of bulk field latex whose viable population had been determined after arrival at the laboratory (see above) and the subsequent changes in population were followed. Two effects were noted: an immediate decline, rather less than expected, and a subsequent multiplication. Three examples are illustrated in Fig. 2.

The immediate lethal effect of ammoniation. The extent of the immediate decline in colony count depended directly on the ammonia concentration (Fig. 2). At about 0·3% (w/w) on the whole latex, a minimum was reached in about 2 hr. On the assumption that this was generally true, 43 of the bulk latex samples, whose count was to be determined (see above), were at once ammoniated to 0·3% (w/w) and the count again determined 2 hr later. Both the fresh latex and the 2 hr ammoniated counts are presented in Table 1. The mean population of the 43 samples without ammoniation was $1{\cdot}88 \pm 0{\cdot}73 \times 10^8$/ml and the mean surviving population after ammoniation was $1{\cdot}99 \pm 0{\cdot}72 \times 10^6$/ml, with a maximum of $3{\cdot}0 \times 10^7$ and a minimum of $4{\cdot}0 \times 10^4$/ml.

The growth of the surviving bacteria. The higher the ammonia concentration the slower was the subsequent growth of the survivors. For the cases illustrated in Fig. 2, the slopes of the linear portions of the graphs are 0·028, 0·017 and 0·013 for the ammonia concentrations 0·43, 0·46 and 0·49% w/w on the water phase, corresponding to mean generation times of 14, 18 and 22 hr respectively. Although these

values of slope, taken in pairs, differ significantly at the 10% level (this is the level customarily adopted in the Malayan industry), a more general survey of wider areas would almost certainly remove this significance—a greater variety of bacteria of more variable resistance would be encountered. As shown in Fig. 2, after the minimum had been reached, growth began with no detectable lag phase and continued at a constant logarithmic rate, which depended on the ammonia concentration, for 10–18 days. Ultimately coagulation or thickening occurred and the population was then of the order of 10^9 cells/ml. Factory and laboratory samples differ in that the former generally show true firm coagulation with syneresis, a clear serum separating later as a distinct phase, while the latter usually thicken to a paste but rarely show true coagulation.

Table 1. *The colony counts of a series of* 43 *samples of fresh latex (at* 3 *p.m.) and ammoniated field latex* 2 *hr after ammoniation to* 0·3% *(w/w) on the whole latex*

Colony counts (10^6/ml)			
Initial	Minimal	Initial	Minimal
3,000·0	10·00	23·0	1·10
310·0	4·00	15·7	0·25
15·0	0·39	5·0	1·64
110·0	1·00	56·0	2·10
250·0	0·24	18·2	0·52
20·0	0·04	30·0	0·054
300·0	0·12	77·0	0·48
350·0	0·50	22·0	0·85
900·0	0·60	37·0	1·13
170·0	0·90	105·0	1·56
800·0	3·00	20·0	0·32
60·0	1·50	23·0	1·27
17·0	1·60	24·0	0·93
4·4	0·82	32·0	2·00
120·0	0·54	67·0	0·75
100·0	30·00	55·0	1·10
14·0	5·00	64·0	1·37
45·0	1·00	17·0	0·58
21·0	0·45	69·0	2·30
160·0	0·52	118·0	0·89
240·0	0·10	170·0	0·69
44·0	1·20		

Arithmetic mean (initial), $1{\cdot}88 \pm 0{\cdot}73 \times 10^8$
Arithmetic mean (minimal), $1{\cdot}99 \pm 0{\cdot}72 \times 10^6$

Ammoniated field latex thus differs from fresh latex in having a smaller bacterial population which grows more slowly. The higher the concentration of ammonia the smaller is the rate of growth.

Ammoniated latex concentrate

The immediate effects of concentration and further ammoniation. In practice, ammoniated field latex (about 0·46% w/w on the water phase) is stored for a limited period (up to 24 hr but in general somewhat less than this) and then centrifuged at about 8,000 *g* to give a concentrate which still has approximately 0·46% (w/w)

ammonia on the water phase but has 0·17% (w/w) ammonia on the whole concentrate. The upper fraction after centrifuging constitutes the concentrate and, as the bacteria tend to sediment in the centrifuge, the concentrate contains fewer bacteria than the aqueous fluid below it. Thus, in a specific instance which is typical of normal operation, an ammoniated field latex containing $4{\cdot}5\times10^5$ viable organisms/ml gave a concentrate with only $2{\cdot}9\times10^3$/ml. In general a reduction to about one-hundredth is effected.

To the resulting concentrate more ammonia is added and this causes the viable bacterial content to fall for a time, the rate and extent of the fall depending on the level of ammoniation. With ammoniation to about 2% (w/w) on the water phase, experience showed that the viable population fell for some 50 hr to a minimum value, which on occasion was below 10/ml.

Table 2. *The changes in the colony counts of a series of* 49 *samples of carefully prepared ammoniated latex concentrate* (2·0% *w/w of ammonia on the water phase*) *during storage for* 2 *days* (*laboratory samples, clean working*)

Colony count (no./ml) after			
3 hr	2 days	3 hr	2 days
200	70·0	5,200	20·0
150	70·0	1,000	10·0
3·5	0·95	20,000	20·0
2,000	3·5	4,000	33·0
20·0	5·0	11,400	—
8·0	6·0	22,900	29.0
5·0	0·1	14,200	—
3·0	10·0	18,600	25·0
7·0	2·0	4,300	14·0
12·0	3·0	20,700	13·0
4·0	5·0	28,000	—
40·0	10·0	16,600	8·0
30·0	8·0	21,000	11·0
30·0	4·0	20,000	9·0
320	42·0	—	11·0
10·0	50·0	4,500	26·0
8·0	4·0	9,700	7·0
350	8·0	11,600	18·0
300	1·5	17,000	6·0
3·5	20·0	11,000	25·0
13,100	20·0	42,000	6·0
—	6·0	57,000	23·0
960	11·0	12,400	2·5
9,000	17·0	18,800	—

Arithmetic mean (3 hr), $9{\cdot}10\pm1{\cdot}84\times10^3$
Arithmetic mean (2 days), $15{\cdot}8\pm2{\cdot}44$

Over a period of 412 days many samples were taken of ammoniated latex concentrate prepared under clean but not sterile conditions. One centrifuge was used throughout and operated in a laboratory, and the final ammonia concentration on the water phase was about 2·0%. The bacterial populations were estimated 3 hr after the final ammoniation and again 2 days later when the minimum count should have been reached. The results, given in Table 2, show what was achieved merely

by careful working. During the latter part of the sampling period (Table 2, cols. 3 and 4) the 3 hr counts were much higher than in the earlier part. This cannot be explained: it was not reflected in unusually high 2 day counts. Tentatively, it is ascribed to a seasonal variation in the types of bacteria in the parent field latices.

Table 3. *Examples of the bacterial content of commercial ammoniated latex concentrate, 4–5 days after preparation*

Colony count (no./ml)				
Large scale				Small scale
190	120	23·0	6,400	37·0
7.0	78·0	10·0	400	4·2
1,400	120	3·5	700	74·0
630	1,300	920	2,000	5,700·0
4·5	27·0	220		
Arithmetic means: $7{\cdot}66 \pm 3{\cdot}40 \times 10^2$				$14{\cdot}5 \pm 5{\cdot}80 \times 10^2$

Such results cannot however be achieved in normal working, where the concentrate becomes reinfected through passing through contaminated pipes and being held in large tanks often contaminated by residues from previously stored concentrate. Table 3 shows the counts obtained after 4–5 days storage on samples of commercial ammoniated concentrate. They are in general much higher than those for the laboratory preparations in Table 2, though the counts are not strictly comparable as they were taken after a longer interval. It was not possible to sample earlier than 4 or 5 days after final ammoniation, because of the time required for transport from the more remote commercial installations.

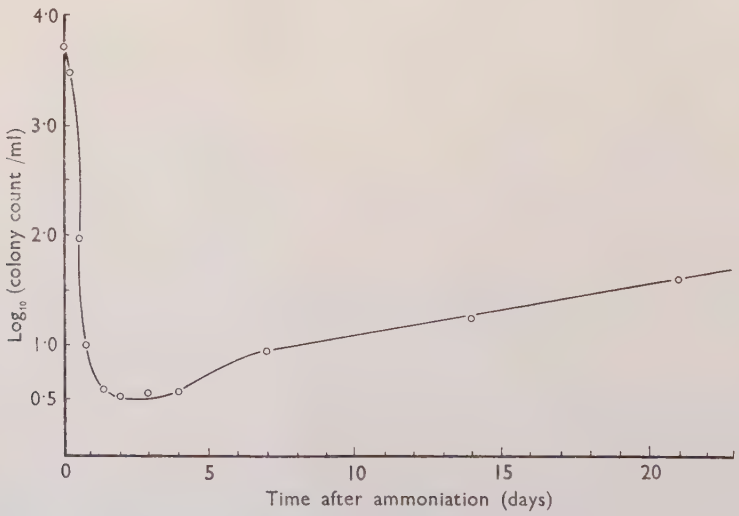

Fig. 3. The general pattern of population decline and rise in ammoniated latex concentrate during storage. Ammonia concentration, 1·86% (w/w) on the water phase.

The growth of the surviving bacteria. Fig. 3 shows the general pattern of slow increase due to the growth of resistant survivors after the initial fall. The case illustrated is that of a hygienically prepared sample with 1·86% ammonia on the water phase. The multiplication, after the relatively sharp rise following the minimum, proceeded logarithmically, with a mean generation time of 6 days. However, this result applies only to concentrate prepared under clean but not sterile conditions. Further, in larger scale production, the residual numbers at the population minimum are often very much larger: in general they are of the order of hundreds to thousands, and in rare cases of the order of tens of thousands to millions.

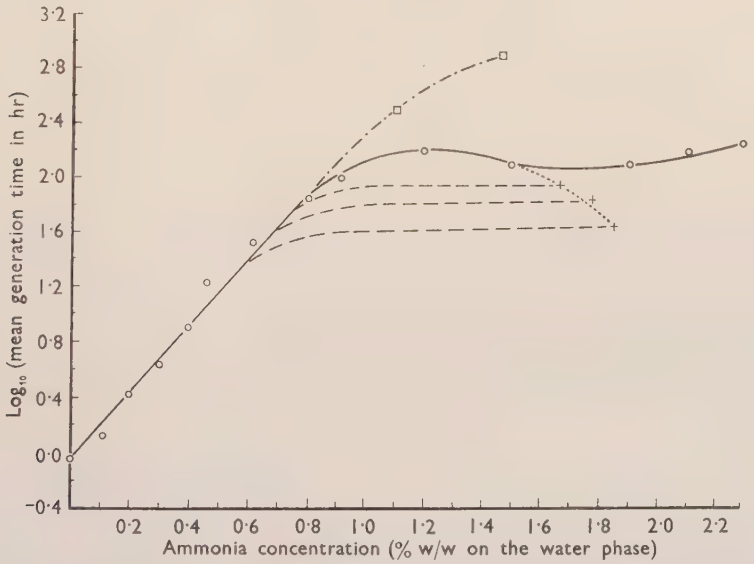

Fig. 4. The general relationship between ammonia concentration and the logarithm of the mean generation time of the bacteria surviving ammoniation, for all stages of latex handling.
Circles, routine laboratory samples; squares, samples prepared under very clean conditions; crosses, commercial samples. Dashes, possible curves representing ordinary commercial working conditions.

The relationship between ammonia concentration and the mean generation time of the surviving bacteria for all types of latex

During the present work, the growth of the surviving bacteria has been followed in a considerable number of latex samples of various kinds and with ammonia concentrations ranging from zero to 2·2% (w/w) on the water phase. In all cases a period of logarithmic growth occurred, from which a mean generation time could be calculated and Fig. 4 shows the relation between ammonia concentration and the logarithm of the mean generation time for all these cases. Among the routine laboratory samples handled without special precautions the logarithm of the mean generation time increased linearly with ammonia concentration up to 0·8–0·9%, and it seems that with samples of latex prepared under very clean conditions this relationship may extend further, i.e. to about 1·1%. Beyond these limits, in both types of

sample, the mean generation time continued to rise, but less rapidly, and in the case of routine laboratory samples it seemed to reach a roughly constant value with 1·2% or more of ammonia. With the few commercial samples examined, however, the mean generation times were distinctly smaller, for given ammonia concentrations, than those given by the routine laboratory samples, and they appeared to decrease progressively with rising concentration from 1·66 to 1·85%. There is further some slight evidence for a similar trend in the routine laboratory samples at a somewhat lower range of concentration.

Discussion

The bacterial population in fresh latex is large in all routine collection and any modification which helps to reduce it or to prevent its rapid proliferation is welcomed, e.g. the cleaning of cups, which reduces the population and increases the lag phase, or the addition of antibacterial anticoagulants. It is not out of place to discuss the benefits which could ensue were the sterilization of cups economic. Assuming a 62% reduction in initial population on substituting a sterile collecting vessel, the increased lag phase would result in the number of bacteria/ml on reaching the factory some 5 hr later being only 17% of that in the normal product. Another distinct gain would be in ammonia resistance. Ammonia is added to the cups as an anticoagulant on a very large number of estates, and although by no means all the ammonia distributed reaches the cups, nevertheless many bacteria are allowed to grow in the presence of low concentrations of ammonia, *c.* 0·02% (w/w), and there is no doubt that a selection of organisms tolerating low ammonia concentrations is achieved. The effect of this selection is cumulative since the latex is not completely removed from the cup after the flow is complete. The author can detect no change in population numbers and no modification of the lag phase with ammonia concentrations below 0·07% (w/w) and can detect no subsequent effect on the mean generation time for the mixed population. There is evidence of a temporary promotion of the growth of micrococci, followed by an enhanced predominance of members of the Lactobacteriaceae. These might conceivably be carried over into the concentrated latex and give rise to the observed growth of resistant bacteria therein. However, there is a much more likely source of contamination on the commercial scale, in the pipelines and storage tanks of the factory, where, under normal unhygienic conditions, bacteria develop continuously in intimate contact with much higher concentrations of ammonia.

Evidence that selection can occur in the field is of several kinds. First, there is the ability of many of the bacteria to survive and grow in latex after ammoniation to 0·3% (w/w), shown above. Second, there is the evidence produced by Taysum (1957) that large numbers of organisms of the family Lactobacteriaceae, especially of the genera *Streptococcus* and *Propionibacterium*, are selected by ammoniation from an initially very mixed population and that species of these genera are known to grow well in ammoniated systems. Third, Taysum (unpublished data) has found that while Malayan soils and badly contaminated river waters, especially the latter, form the principal sources of bacterial contamination of field latex, the organisms in those habitats are very sensitive to ammoniation to 0·3% (w/w). In these soils,

a reduction of two orders can often be achieved in the first hour after ammoniation to 0·3% (w/w). But these organisms are not accustomed to the presence of ammonia, while those which grow in ammoniated systems are. Fourth, as pointed out above (Fig. 4), the growth of the surviving bacteria in ammoniated field latex and ammoniated latex concentrate may actually be faster under certain conditions at higher than at lower ammonia concentrations.

Some important results were obtained by a statistical analysis of the data of Fig. 1, not apparent from visual examination. As previously, a 10% level of significance was adopted. The lag and logarithmic phases of the growth curve may be regarded as compounded of two linear portions, or of two linear portions connected by a curve. By adopting the first definition it was possible to use all the colony count data in the analysis; with the second definition certain values were disregarded because they seemed to fall on the curved portion. Calculation of the slopes of the lines representing the two phases led to a significant negative correlation between initial count and the length of the lag phase in both cases (at the 10% level). Calculations from all the data produced a significant positive correlation between duration of log phase and initial count, whereas when certain data were disregarded this significance disappeared.

Another fact which emerged was that the higher the initial population, the lower was the maximum population recorded: this is significant and may mean that the growth of bacteria in latex may be finally inhibited by the toxic products of previous metabolism, rather than by limited amounts of substrates.

Ammoniated field latex is characterized by the presence of large numbers of organisms which are capable of continued growth under alkaline conditions, the rate being such that in industrial practice about $1\frac{1}{4}$ generations occur before the latex is centrifuged. The volatile fatty acid content of the field latex rises by 0·04–0·06 units from about 0·03 units.

In ammoniated latex concentrate the mean generation time of the surviving organisms was found to be sensitively dependent upon the concentration of ammonia present. Two other factors, however, seem to be important on the industrial scale. First, it is difficult, often impossible, to preserve latex concentrate which has been prepared from a large bulk of field latex in which bacterial proliferation to a high level has already occurred, because ammonia resistant strains have developed. They can frequently be isolated from such concentrate. Second, evidence has accumulated that in most centrifuging plant resistant bacteria are introduced—the conditions being grossly unhygienic. Thus even where no fault can be found with the method of collection or the speed of ammoniation, and where the viable population of the field latex is not abnormally high, the concentrate still becomes inoculated with resistant bacteria. In many cases bacterial strains identical with those of infected concentrate have been detected in scrapings from factory structures.

A further factor may be of considerable importance in large, commercial scale working. This is that the deep storage condition of most bulk concentrate would favour both the selection of anaerobic bacteria and the operation of the Pasteur effect; the latter would result in enhanced glycolysis by all cells and consequently accelerated degradation, above that which the level of infection might suggest.

Indeed, the aeration of fully ammoniated concentrate is at present a fairly effective method of reducing the viable population. This reduction, which can be followed quantitatively, indicates not only the considerable sensitivity of anaerobes to aeration processes but also that these organisms are effectively plated for concentrated latex. In the case of poorly prepared commercial concentrate, however, the redox potential may already be so low as to be beyond the range for even strict anaerobes, and in this case slight aeration, such as occurs in pumping from a deep storage tank to a deep rail tanker may bring the redox potential to a level ideal for the proliferation of facultative anaerobes. There is some evidence that this does occur in practice, with undesirable consequences.

In Fig. 4 an alternative interpretation of bacterial resistance in commercial samples of ammoniated latex concentrate has also been presented. It is possible, though difficult to justify, that the relatively low mean generation times of commercial samples result from the existence of a family of relationship curves which become roughly parallel to the abscissa for higher ammonia concentrations. There is, of course, no method of verifying this alternative presentation since it is grossly uneconomic to imperfectly preserve large volumes of commercial field latex and concentrate.

Finally, it must be remembered that the viable counting technique underestimates to some degree the true number of viable cells because of clumping and the possible failure of some species to grow. It also takes no account of dead cells. Now in ammoniated field latex with 0·46% (w/w) on the water phase, the colony counts indicated an average mean generation time of 18 hr. But this may be an unduly high estimate. There is evidence, to be reported in a later paper, that the true average mean generation time is in fact 6–8 hr, the observed value of 18 hr resulting in part from the death of a large proportion of the progeny. Further, during the bulk shipment of concentrate, the viable population does not continue to rise but remains almost constant or falls very slowly, and this almost certainly results from an equilibrium between growth and death, since it might be expected that death would be rapid in this highly inhibitory system. These dead bacteria may still accomplish considerable enzymic degradation of latex constituents during storage and shipment.

Although these results were obtained from limited areas of production and limited volumes of latex, the majority of the latices being derived from the Rubber Research Institute Experiment Station, Sungei Buloh, and it remains to be established that the general picture presented is true for most of the commercial establishments in Malaya, it is thought that the data provide a sound basis for action designed to combat bacterial infection of *Hevea* latex and concentrate.

The author wishes to thank Mr. A. S. Dhillon for active co-operation and Messrs. Graham, Gopal and Kandasamy for organizing the supply of many of the samples. Thanks are due to Mr. Westgarth and Mr. Iyer for statistical analyses.

The work described in this paper forms part of the programme of research on the bacteriology of *Hevea* latex undertaken by the Board of the Rubber Research Institute of Malaya.

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THE EFFECT OF VARIOUS WATER BACTERIA ON THE GROWTH OF *ESCHERICHIA COLI* I IN MACCONKEY'S BROTH AT 37° AND 42°

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SUMMARY: The growth of a streptomycin resistant strain of *Escherichia coli* I in MacConkey's broth at 37° or 42° was not inhibited by water organisms of the genera *Micrococcus*, *Proteus*, *Achromobacter* and *Pseudomonas*, but the presence of large numbers of other members of the coli-aerogenes group resulted in retardation or inhibition of its growth.

The *Escherichia coli* test, carried out by inoculation of MacConkey's broth with incubation for 48 hr at 44°, is considered to be a reliable index of excretal pollution in countries with temperate climates. The test is officially sanctioned in Britain and it has been shown that about 85% of waters giving a positive presumptive coli-aerogenes test contain *E. coli* I (Taylor, 1949; Clegg, 1952; Thomas *et al.* 1953). In the tropics, a fairly high proportion of the coli-aerogenes organisms in water are often of the intermediate or aerogenes-cloacae types, so that the detection of *E. coli* becomes important. The problem then arises as to the extent of inhibition of *E. coli* by other bacteria commonly found in water, and in an investigation of this problem the experiments described below were carried out.

Experimental Methods

Media. Liquid; MacConkey's broth, lactose broth and peptone water. Solid; MacConkey's agar, lactose agar and citrate agar. The latter contained, in addition to the usual components of citrate agar, 1 ml of meat broth in each 200 ml. On this medium the citrate-negative *E. coli* will grow and form small discrete translucent colonies. Citrate-positive organisms form opaque colonies and the two types are easily distinguishable, thus permitting differential counts.

Micro-organisms. Most of the bacteria used were isolated in this laboratory from different water sources, including surface and deep waters, swimming baths and pools. The *E. coli* strains used were of human origin. One strain of *E. coli* I was isolated from a patient treated with streptomycin; it was streptomycin resistant and was therefore conveniently used as the test organism whose inhibition was to be studied.

Procedure. The streptomycin resistant *E. coli* I was grown in peptone water for 24 hr at 37° and diluted to 10^{-4} in sterile water. A loopful of this (10^{-3} ml) was then transferred to 25 ml of MacConkey's broth or sometimes to 25 ml of peptone water, well shaken, and 2·5 ml quantities distributed in sterile tubes. Each 2·5 ml thus received 10^{-8} ml of *E. coli* culture and the inoculation level varied from 2–10 cells/ml

in different experiments. One tube was kept as a control while the rest received various numbers of other bacteria through the addition of a loopful of a suitable sterile water dilution of a peptone water culture grown at 37° for 24 hr, or a loopful of undiluted culture. The tubes were then incubated at 37° or 42° as desired, and at intervals 2–4 loopfuls from each were streaked on to citrate agar or citrate agar containing dihydrostreptomycin (100 μg/ml) and sometimes also on lactose agar for counting some non-coli-aerogenes bacteria. After 24–48 hr incubation the colonies of the various kinds of bacteria were counted. If large numbers were expected from a culture, it was diluted in sterile water before the loopfuls were streaked.

Results

The influence of bacteria other than the coli-aerogenes group

Details of the progress of mixed cultures of *E. coli* I and organisms not belonging to the coli-aerogenes group are given in Table 1. *E. coli* was clearly not inhibited by the presence initially of large numbers of organisms of species of the genera *Micrococcus* and *Achromobacter*; in fact these organisms disappeared within 24 hr, presumably as a result of the presence of bile salts and the accumulation of acid in the medium. This was confirmed by comparing the simultaneous growth of the mixed cultures in MacConkey's broth, lactose broth and peptone water. The *Proteus* sp., being also sensitive to bile salts, did not significantly affect the growth of *E. coli*, and was present in small numbers only after 24 hr. It is interesting to note that the *Pseudomonas aeruginosa*, which is bile tolerant, did not affect the growth of *E. coli*; even when there were initially about 10^5 as many *Ps. aeruginosa* as *E. coli* (tube 9) the latter grew almost as well as in the control during the first 24 hr of the experiment. In contrast to the species of *Micrococcus* and *Achromobacter*, *Ps. aeruginosa* did not disappear within 24 hr.

Table 1. *The growth of* E. coli *I in MacConkey's broth in pure culture and in association with micro-organisms other than coli-aerogenes*

Tube no.	Inoculum (ml /2.5 ml) of 24 hr cultures of:		No. of organisms (/10^{-3} ml) developing after incubation for:							
			5½ hr		12 hr		24 hr		48 hr	
	E. coli	Test culture	*E. coli*	Test culture	*E. coli*	Test culture	*E. coli*	Test culture	*E. coli*	Test culture
1	10^{-8}	None	14	—	160,000	—	384,000	—	—	—
2	10^{-8}	*Micrococcus* (10^{-6})	16	400	105,000	500	300,000	0	—	—
3	10^{-8}	*Micrococcus* (10^{-3})	20	230,000	166,000	4,500	430,000	0	—	—
4	10^{-8}	*Achromobacter* (10^{-6})	—	—	182,000	1,125	450,000	0	—	—
5	10^{-8}	*Achromobacter* (10^{-3})	—	—	180,000	3,937	435,000	0	—	—
6	10^{-8}	*Proteus* (10^{-6})	—	—	166,000	<500	305,000	<500	—	—
7	10^{-8}	*Proteus* (10^{-3})	—	—	40.000	76.000	247,000	47,500	—	—
8	10^{-8}	*Pseudomonas aeruginosa* (10^{-6})	—	—	100,000	50,000	420,000	145,000	20,000	0
9	10^{-8}	*Pseudomonas aeruginosa* (10^{-3})	—	—	138,000	220,000	300,000	395,000	15,000	0

The influence of other coli-aerogenes bacteria

Table 2 shows the results obtained with *Klebsiella cloacae*. This organism produced much less acid in MacConkey's broth than other members of the coli-aerogenes group. The pH of a culture incubated for 24 hr was 6·55, compared with 5·0 for *K. aerogenes*,

4·8 for *Citrobacter freundii* and 4·6 for *E. coli* I and *E. coli* II. Initially there were 10^2, 10^3 and 10^5 times as many *K. cloacae* as *E. coli*. After 6 hr the latter had grown as well as in the control where there were only 10^2 times as many *K. cloacae*, but with the 10^3 ratio there was a slight retardation and at the 10^5 ratio a marked retardation. After $10\frac{1}{2}$ hr incubation *E. coli* was inhibited in all cases, as compared with the control, the degree of inhibition being according to the initial ratio of *K. cloacae*. However, after 24 hr incubation the inhibition was largely overcome, though still most evident where the initial ratio of *K. cloacae* had been highest. It may be concluded, therefore, that *E. coli* cannot be prevented from growing by *K. cloacae*, even when the sample comes from a water excessively contaminated with that organism.

Table 2. *The growth of* E. coli *I in MacConkey's broth in pure culture and in association with* K. cloacae

Tube no	Inoculum (ml/2·5 ml) of 24 hr cultures of:		No. of organisms (/10^{-3} ml) developing after incubation for:					
			6 hr		$10\frac{1}{2}$ hr		24 hr	
	E. coli	*K. cloacae*	*E. coli*	*K. cloacae*	*E. coli*	*K. cloacae*	*E. coli*	*K. cloacae*
1	10^{-8}	None	54	—	50,000	—	390,000	—
2	10^{-8}	10^{-6}	53	500	6,500	215,000	267,000	135,000
3	10^{-8}	10^{-5}	44	25,500	1,500	195,000	300,000	230,000
4	10^{-8}	10^{-3}	14	125,500	178	235,000	227,000	300,000

Table 3. *The growth of* E. coli *I in MacConkey's broth in pure culture and in association with* K. aerogenes *I*

Tube no.	Inoculum (ml/2·5 ml) of 24 hr cultures of:		No. of organisms (/10^{-3} ml) developing after incubation for:						Amount (ml) of N/50 NaOH to neutralize 2·5 ml of culture after
			$10\frac{1}{2}$ hr		24 hr		48 hr		
	E. coli	*K. aerogenes*	*E. coli*	*K. aerogenes*	*E. coli*	*K. aerogenes*	*E. coli*	*K. aerogenes*	48 hr
1	10^{-8}	None	10,000	—	360,000	—	5,000	—	1·7
2	10^{-8}	10^{-6}	1,500	95,000	26,250	190,200	7,000	26,000	1·4
3	10^{-8}	10^{-3}	16	172,000	187	265,000	2,800	22,000	1·2

The results obtained with *K. aerogenes* are given in Tables 3 and 6. No inhibition in the growth of *E. coli* was found after 5 hr (Table 6, tube 4) but there was a marked retardation after $10\frac{1}{2}$ hr and 24 hr and this was especially severe with the highest ratio of *K. aerogenes* (Table 3, tube 3). It is thought that this retardation, in contrast to the temporary retardation brought about in mixed culture with *K. cloacae* (Table 2), may have been largely due to greater acid production by *K. aerogenes*. To neutralize 2·5 ml of a 24 hr culture of *K. aerogenes* in MacConkey's broth 0·8 ml of N/50 NaOH was required: but a similar culture of *K. cloacae* needed only 0·2 ml. Several strains of *K. aerogenes* were examined, and there appeared to be a correlation between the amount of acid produced and the degree of inhibition of *E. coli*. However, after 48 hr the numbers of *E. coli* were not greatly different from those in the control, where considerable death of *E. coli* had occurred.

Tables 4 and 6 show the results obtained with *Citrobacter freundii*. With this organism there was no inhibition of *E. coli* during the first 5 hr in MacConkey's broth (Table 6, tube 5), but after $6\frac{1}{2}$ hr (Table 4) there was a slight inhibition in tube 2 (ratio 10^2) and a strong inhibition in tube 3 (10^5 ratio). The latter persisted through the experiment, with no development at all of *E. coli*. It is probable that acid formation is one of the causes of the *Cit. freundii* inhibition of *E. coli*, since in a parallel experiment in peptone water the inhibitory effect was much smaller (Table 4).

Table 4. *The growth of* E. coli *I in MacConkey's broth and peptone water in pure culture and in association with* Cit. freundii

Medium and tube no.		Inoculum (ml/2·5 ml) of 24 hr cultures of:		No. of organisms (/10^{-3} ml) developing after incubation for:					
				$6\frac{1}{2}$ hr		$11\frac{1}{2}$ hr		27 hr	
		E. coli	*Cit. freundii*	*E. coli*	*Cit. freundii*	*E. coli*	*Cit. freundii*	*E. coli*	*Cit. freundii*
MacConkey's broth	1	10^{-8}	None	172	—	115,400	—	407,200	—
	2	10^{-8}	10^{-6}	144	46,600	1,443	209,800	1,400	273,000
	3	10^{-8}	10^{-3}	7	380,000	6	308,000	2	205,000
Peptone water	4	10^{-8}	None	—	—	228,000	—	399,000	—
	5	10^{-8}	10^{-6}	—	—	6,327	251,000	124,300	400,000
	6	10^{-8}	10^{-3}	—	—	100	482,000	4,660	431,000

Table 5. *The growth of* E. coli *I in MacConkey's broth in association with* K. aerogenes *I or* Cit. freundii *I at* 37° *and* 42°

Tube no.	Incubation temp. (°C)	Inoculum (ml/2·5 ml) of 24 hr cultures of:			No. of organisms (/10^{-3} ml) developing after incubation for:					
					$6\frac{1}{2}$ hr		$10\frac{1}{2}$ hr		24 hr	
		E. coli	Test organism		*E. coli*	Test organism	*E. coli*	Test organism	*E. coli*	Test organism
1	37	10^{-8}	None		18	—	10,500	—	360,000	—
2	42	10^{-8}	None		40	—	27,000	—	250,000	—
3	37	10^{-8}	*K. aerogenes* I	10^{-6}	11	350	1,000	165,000	11,000	300,000
4	42	10^{-8}		10^{-6}	26	75	4,000	94,500	10,500	60,000
5	37	10^{-8}		10^{-3}	1	240,000	9	255,000	73	240,000
6	42	10^{-8}		10^{-3}	7	57,000	35	107,500	220	80,000
7	37	10^{-8}	*Cit. freundii* I	10^{-6}	28	500	3,500	80,000	50,500	390,000
8	42	10^{-8}		10^{-6}	17	125	4,000	500	225,000	<500
9	37	10^{-8}		10^{-3}	11	255,000	28	355,000	28	350,000
10	42	10^{-8}		10^{-3}	35	2,000	61,000	750	158,000	<500

Comparison of the inhibitory effects of K. aerogenes *I and* Cit. freundii *I on* E. coli *I at* 37° *and* 42°

It is well known that *E. coli* is generally more resistant to 42° than the other coli-aerogenes bacteria, and the incubation of MacConkey's broth at 42° instead of 37° often helps to detect *E. coli*, especially when its numbers are small compared with those of other coli-aerogenes organisms present. Experiments were therefore done

at both 37° and 42° with *E. coli* in association with *K. aerogenes* I and *Cit. freundii* I. As shown in Table 5, the *Cit. freundii* was much more sensitive in its growth to 42° than was *K. aerogenes* I (compare tubes 3 and 4 with 7 and 8, and 5 and 6 with 9 and 10). In agreement with this, the inhibitory effect of *K. aerogenes* I at the 10^5 ratio at 42° (tube 6) was much more marked than that of *Cit. freundii* at the same ratio and temperature (tube 10), though at 37° the inhibitions were both equally severe (tubes 5 and 9).

The combined effect of several coli-aerogenes organisms on the growth of E. coli

Since several organisms may be present together with *E. coli* in a tube of a presumptive test, the growth of the latter in association with various combinations of bacteria was studied. The results are shown in Table 6, where only the development of the *E. coli* is recorded. Tubes 2–5 confirmed the results of the previous tests with only one organism associated with *E. coli* I, and in addition tube 6 showed that *E. coli* II, when 10^5 times as numerous as *E. coli* I, could severely inhibit the latter. When two or more associated organisms were present with *E. coli* I, the general rule was that the strongest inhibitor among them exerted its usual effect. In the case of *Cit. freundii* I, *K. aerogenes* I and *E. coli* II this left little room for further inhibition by the other organisms, and while the *E. coli* I counts in the cultures incubated for 24 hr were generally even lower when additional organisms were present (cf. tubes 4 and 8, 5 and 9, 6 and 10), the results cannot be regarded as unequivocally indicating a cumulative effect.

Table 6. *The growth of* E. coli *I in MacConkey's broth in pure culture and in association with different bacteria*

Tube no.	Organisms inoculated*	No. of *E. coli* I (colonies/10^{-3} ml) after incubation for:		
		5 hr	12 hr	24 hr
1	A	2·5	165,000	462,000
2	A, B	3	40,250	500,000
3	A, C	1	1,250	187,000
4	A, D	2·5	30	85
5	A, E	2·5	35	45
6	A, F	3	15	2
7	A, B, C	3·5	750	317,000
8	A, B, C, D	3	19	55
9	A, B, C, D, E	2	11	20
10	A, B, C, D, E, F	**0·5**	**4**	**1**

* A, *E. coli* (10^{-8} ml/2·5 ml). *B, Ps. aeruginosa.* C, *K. cloacae.* D, *K. aerogenes* I. E, *Cit. freundii* I. F, *E. coli* II. Organisms B–F were all inoculated at 10^{-3} ml/2·5 ml.

Discussion

A retardation in the growth of *E. coli* I in mixed cultures was not noted, in these experiments, with non-coli-aerogenes bacteria such as *Pseudomonas*: but it was very marked with coli-aerogenes organisms. This delay did not occur in the first 5 hr of common growth, when the inhibitor was presumably in the logarithmic phase, but

only later when it had probably closely approached or reached the stationary phase. The larger the inoculum the more rapidly would this phase be reached by the inhibitor, and this is in accordance with the fact that in mixed culture the retardation of *E. coli* was first manifest in the tube with the largest inoculum of inhibitor.

In the experiments where *E. coli* was grown in association with *K. cloacae*, the pH of the MacConkey's broth altered only slightly during the period when the latter was growing. But after that the conditions were suitable for the further development of *E. coli*, and in 24 hr that organism attained a level similar to that reached in pure culture. In mixed culture with *K. aerogenes* or *Cit. freundii* the further multiplication of *E. coli* was delayed: this can be explained by the accumulation of acid in the medium. *E. coli* I is readily inhibited by *E. coli* II, presumably because of rapid acid production, but this is not of practical importance because *E. coli* II is relatively rare in water. After 2–3 days most of the *K. aerogenes* or *Cit. freundii* cells died and it often became possible to detect the few *E. coli* organisms. Thomas *et al.* (1953) observed that isolation of *E. coli* was often easier after 48 hr than after 24 hr. Naturally, in a mixed population in MacConkey's broth the coli-aerogenes organisms with the greatest tolerance to acid have the best chance of surviving.

It can be concluded from our experiments that most of the miscellaneous organisms which are commonly found in water do not unduly hinder the recovery of *E. coli*. Only those strains of the coli-aerogenes group which produce large amounts of acid in MacConkey's broth can prevent the isolation of *E. coli* I, and then only when they are present in large numbers. Since inhibitory effects have been demonstrated only when the inhibitor had a considerable numerical superiority over *E. coli* I, the question arises whether such effects are likely to occur in practice. To answer this, further work is required to determine how low the proportion of the inhibitory organism can be brought before the effect vanishes or becomes of no practical significance, and to determine the relative incidence of *E. coli* I and the other organisms in natural waters.

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THE USE OF STORED SUSPENSIONS OF *ESCHERICHIA COLI* I IN THE EVALUATION OF BACTERICIDAL ACTION

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SUMMARY: Suspensions of *Escherichia coli* I, consisting of washed cells suspended in a phosphate buffer solution, maintained a higher viability and resistance to phenol than suspensions either of unwashed cells or of washed cells suspended in water. When stored for 5 weeks at room temperature, variations in their extinction times on exposure to aqueous phenol solutions were not significantly greater than variations with suspensions freshly prepared for each determination. Loss of resistance of a stored suspension to phenol, roxenol, lysol and potassium laurate was roughly parallel. Conditions of culture of the bacteria influenced the survival of suspensions, but the results indicated that pronounced differences may only be found in suspensions prepared from young cultures. The use of stored suspensions in the routine evaluation of bactericides is recommended.

The problem of obtaining consistent evaluations of bactericidal activity between different workers and laboratories is well known. Cook & Wills (1956*a*,*b*) drew attention to several factors which influence the reproducibility of estimates of extinction times of *Escherichia coli* to phenol; since a design of experiment was used which could account for sampling variations, this work, like that of Garvie (1955) and Garvie & Clarke (1955), constituted the first adequate attempts to investigate these factors. Following the report by Cook & Steel (1955) that aqueous suspensions of *E. coli* could be stored with substantial maintenance of viability, Cook, Steel & Wills (1956) further studied this phenomenon in relation to resistance to bacteriostatic materials and reproducibility of extinction times and concluded, among other points, that suspensions of *E. coli* I stored at low temperatures (4–10°) would give reasonably constant resistance to a bactericidal concentration of phenol over a period of two or three months. In this study, however, no information was given on the relative merits of washed and unwashed cells, of suspending fluids other than water, or of the significance of different methods of culture, and phenol was the only bactericide used. Information on these points forms the subject of the present communication.

Methods

Bacterial suspensions. The organism used was a laboratory strain of *Escherichia coli* I, 44° positive, formerly NCTC 5933. Suspensions, unless otherwise stated, were prepared from 24 hr cultures on peptone agar slopes as described by Cook & Wills (1956*b*). After vigorous shaking with sterile glass beads, the suspensions were centrifuged at 3500 rev/min for 1 min and the supernatant withdrawn. Washed suspensions

were prepared by centrifuging for 25 min, resuspending the cells in standardized volumes of water and repeating this process three times. Each suspension, whether unwashed, or washed and suspended in water or a different solution, was standardized as described by Cook & Wills (1956*b*) to contain approximately 2×10^9 organisms/ml. They were stored in 60 ml glass-stoppered bottles.

Testing procedure. Viable counts of the suspensions were estimated by the surface overdried plate method of Miles & Misra (1938), using the techniques of Cook, Steel & Wills (1956). Extinction times at 20° on exposure to solutions of bactericides were determined by the method of Berry & Bean (1954), subject to the modifications and method of expressing the result previously described (Cook & Wills, 1954). Each estimate of extinction time was obtained from between 10 and 20 replicate determinations and the result expressed as the 'mean single survivor time' (Mather, 1949). The extinction times were subjected to an analysis of variance, using a two factor analysis for each suspension type with variances estimated for between suspensions and between storage times.

Results

Comparison of the resistance of unwashed and washed suspensions in different suspending fluids to phenol

Four different batches of suspension were prepared over a period of one month. The first batch was prepared from the 14th of a series of daily subcultures and further batches were made at intervals of about 10 days, the last being from the 45th subculture. Each of the first three batches was divided into three equal portions: one was left unwashed, the second was washed and resuspended in distilled water and the third was washed and resuspended in 0·1 M phosphate buffer, pH 7·0. The fourth suspension was divided into four portions, the fourth part being resuspended in 0·1 M citrate buffer, pH 6·4. All 13 suspensions were stored at room temperature (18–20°).

Viable counts and extinction times on exposure to 1·1% aqueous phenol solution were determined for each suspension during the first, third and fifth week of storage. The first determinations were made as soon as possible after preparation of the suspension, either on the same day or the next day, and the second and third determinations were made as closely as possible to the 21st and 35th day of storage. The results are shown in Tables 1 and 2 and the statistical analyses of the results are summarized in Table 3. Noticeably, the second washed, unbuffered suspension suffered a much greater loss of viability and phenol resistance on storage than any other suspension studied; hence no analysis was possible. The reason for this loss is not known: such rapid decay had not been encountered by the authors before with stored suspensions of this organism.

Tables 1 and 2 show that (*a*), viability was best maintained at room temperature in washed, phosphate-buffered suspensions and worst in unwashed suspensions (although there was a gross anomaly with the second washed, unbuffered suspension); (*b*), retention of viability in citrate buffer was inferior to that in phosphate buffer;

(*c*), initial extinction times tended to be highest with the unwashed suspensions, intermediate with the washed buffered suspensions and lowest with the washed, unbuffered suspensions; (*d*), extinction times after storage appeared to be best maintained with washed, buffered suspensions, and (*e*), there was little difference between the initial viable counts of different suspensions, but a considerable difference between their initial extinction times, this being in agreement with previous findings (Cook & Wills, 1956*b*).

Table 1. *The viabilities of four batches of four different suspensions of* E. coli *stored at room temperature* (18–20°)

Kind of suspension	Age (weeks)	Viable count/ml on suspension batch			
		1	2	3	4
Unwashed cells	0	$1{\cdot}94\times10^{9}$	$1{\cdot}85\times10^{9}$	$2{\cdot}01\times10^{9}$	$1{\cdot}89\times10^{9}$
	3	$3{\cdot}60\times10^{8}$	$6{\cdot}66\times10^{8}$	$9{\cdot}60\times10^{7}$	$1{\cdot}96\times10^{8}$
	5	$1{\cdot}88\times10^{8}$	$3{\cdot}73\times10^{8}$	$3{\cdot}84\times10^{7}$	$1{\cdot}56\times10^{7}$
Washed cells	0	$1{\cdot}98\times10^{9}$	$2{\cdot}03\times10^{9}$	$2{\cdot}03\times10^{9}$	$1{\cdot}93\times10^{9}$
	3	$1{\cdot}31\times10^{9}$	$1{\cdot}26\times10^{5}$	$1{\cdot}43\times10^{9}$	$1{\cdot}76\times10^{9}$
	5	$1{\cdot}06\times10^{9}$	$5{\cdot}70\times10^{4}$	$8{\cdot}40\times10^{8}$	$1{\cdot}11\times10^{9}$
Washed cells in phosphate buffer, pH 7·0	0	$1{\cdot}98\times10^{9}$	$1{\cdot}95\times10^{9}$	$1{\cdot}97\times10^{9}$	$1{\cdot}90\times10^{9}$
	3	$1{\cdot}85\times10^{9}$	$1{\cdot}85\times10^{9}$	$1{\cdot}82\times10^{9}$	$1{\cdot}73\times10^{9}$
	5	$1{\cdot}68\times10^{9}$	$1{\cdot}64\times10^{9}$	$1{\cdot}06\times10^{9}$	$8{\cdot}22\times10^{8}$
Washed cells in citrate buffer, pH 6·4	0	—	—	—	$1{\cdot}49\times10^{9}$
	3	—	—	—	$1{\cdot}51\times10^{8}$
	5	—	—	—	$1{\cdot}45\times10^{8}$

The three two-factor analyses of variance between storage times and between suspensions (Table 3) indicate that only with suspensions stored in phosphate buffer were the variance estimates both between suspensions and between storage times not significant at the 5% probability level when compared with the residual variance estimate. With such suspensions, therefore, determinations of extinction times to phenol over a period of five weeks, or longer if stored at a lower temperature, should show no greater variability than with suspensions freshly prepared for each determination (Cook, Steel & Wills, 1956).

Table 2. *The extinction times, to* 1·1% *aqueous phenol at* 20°, *of four batches of three suspensions of* E. coli *at room temperature* (18–20°)

Kind of suspension	Age (weeks)	Extinction time (min) of suspension batch			
		1	2	3	4
Unwashed cells	0	29·2	36·0	33·4	43·4
	3	27·7	31·9	24·6	26·2
	5	24·4	22·7	20·3	24·4
Washed cells	0	24·5	27·1	24·6	27·2
	3	25·2	<10	16·0	17·4
	5	21·2	< 1	16·2	16·1
Washed cells in phosphate buffer, pH 7·0	0	26·7	35·1	25·8	34·1
	3	27·6	22·4	18·3	26·7
	5	25·5	23·1	25·7	20·0

Table 3. *Analysis of variance between suspensions and between storage times for extinction times of each suspension type, from data in Table 2*

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance ratio	*P*
Unwashed suspensions					
Suspensions	3	55·51	18·50	1·21	>0·2
Storage times	2	322·05	161·03	10·50	0·01
Residual	6	92·00	15·33		
Totals	11	469·56			
Washed suspensions in distilled water					
Suspensions	2	52·07	26·04	5·55	0·05–0·1
Storage times	2	112·19	56·10	11·95	0·02
Residual	4	18·77	4·69		
Totals	8	183·03			
Washed suspensions in phosphate buffer					
Suspensions	3	28·28	9·43	—	—
Storage times	2	122·01	61·01	3·09	>0·1
Residual	6	118·63	19·77		
Totals	11	268·92			

The use of other bactericides

The bactericides chosen included the two common phenolic disinfectant fluids, lysol and roxenol. The samples of lysol and roxenol were prepared with castor oil from the same source, and both fluids complied with the requirements of the British Pharmacopoeia. A third bactericide used was potassium laurate, chosen as a representative detergent, with which experience had previously been gained (Berry, Cook & Wills, 1956). Preliminary determinations showed that concentrations of 0·45% of roxenol, 0·66% of lysol and 1·67% of potassium laurate would give extinction times with freshly prepared suspensions within the range of 20–40 min.

The growths from a sufficient number of agar slopes were used to prepare two 60 ml bottles full of a washed suspension in pH 7·0 phosphate buffer, the conditions for optimal stability. One bottle was stored at 4°, and the other at 37°. Viable counts were determined immediately after preparation and at intervals during the following five weeks. As soon as possible in the first week, and again in the third and fifth weeks of storage, the extinction times of each suspension on exposure to solutions of phenol, lysol, roxenol and potassium laurate at 20° were determined. The results are shown in Tables 4 and 5, and Table 6 lists the extinction times after storage for three and five weeks expressed as percentages of the initial extinction times. While

Table 4. *The viability of buffered suspensions of* E. coli *stored at* 4° *and* 37°

Storage temperature (°C)	Viable counts (/ml) after storage for (days)				
	0	4	10	17	31
4	$1·98\times10^9$	$1·95\times10^9$	$1·88\times10^9$	$1·70\times10^9$	$1·64\times10^9$
37	$1·98\times10^9$	$6·90\times10^8$	$1·64\times10^8$	$2·84\times10^7$	$1·36\times10^6$

the results show a general decrease in the extinction times with storage (Table 5), the percentages of decrease are (Table 6) similar for all four bactericides, namely, 71–81% after three weeks and 64–73% after five weeks storage at 4°, and 15–29% after three weeks and 13–25% after five weeks storage at 37°. Thus resistance to all the bactericides studied was maintained to roughly the same extent, and estimates of extinction time expressed as a ratio of bactericidal action to that of a reference substance can be expected to be maintained very consistently with a stored suspension.

Table 5. *The extinction times to different bactericides of buffered suspensions of* E. coli *stored at* 4° *and* 37°

Bactericide and concentration (%)		Extinction times (min) when stored at					
		4° for (weeks)			37° for (weeks)		
		0	3	5	0	3	5
Phenol	1·1	39·2	27·9	28·6	39·2	6·1	5·2
Lysol	0·66	44·6	36·2	28·4	44·6	13·2	10·0
Roxenol	0·45	21·6	16·4	14·3	21·6	5·8	3·1
K laurate	1·67	32·3	25·4	20·5	32·3	—	7·9

Table 6. *The extinction times to different bactericides of buffered and stored suspensions of* E. coli, *expressed as percentages of initial values (see Table* 5)

Bactericide and concentration (%)		Percentage extinction times when stored at			
		4° for (weeks)		37° for (weeks)	
		3	5	3	5
Phenol	1·1	71·2	73·0	15·4	13·4
Lysol	0·66	81·2	63·7	29·6	22·4
Roxenol	0·45	75·9	66·2	26·6	14·3
K laurate	1·67	78·6	63·5	—	24·6

The viability during storage of suspensions of washed cells in distilled water, prepared from organisms grown under different cultural conditions

Since wide variations in the heat resistance of suspensions of *E. coli* prepared from cultures grown under different conditions have been demonstrated (Wills, 1957), it seemed desirable to investigate the storage properties of such suspensions. The organisms were grown (*a*) in a simple peptone-salt liquid medium, aerated and agitated during growth, or left static and not aerated; (b), on plates and slopes of solidified medium, either overdried or not dried; (c), on plates with lids filled with soda lime, or (*d*), on plates with lids sealed with cellulose tape. The aerated liquid media were also incubated for varying lengths of time (12–65 hr) before harvesting the cells, so that conditions representing differences in aeration, duration of incubation, exclusion of carbon dioxide during growth and surface moisture of the solid medium were established. All cultures were incubated at 37°, and the suspensions

were of washed cells in distilled water. They were filled into 60 ml glass-stoppered bottles and stored at room temperature exposed to sunlight. Viable counts were determined initially and after storage for 1, 2 and 3 months. The results, which are given in Table 7, indicate that the maintenance of viability in washed suspensions of *E. coli* stored at room temperature was little affected by the alteration in cultural conditions, the viable counts of suspensions prepared from 24 hr growths on solid media ranging from about 8×10^5 to 7×10^6/ml after 3 months. The age of the aerated liquid culture from which the suspension was prepared appears to have had a greater influence on the retention of viability, particularly in the case of the shortest incubation period of 12 hr. In the case of non-aerated liquid cultures, suspensions were prepared only after incubation for 24 and 48 hr, and viable counts were performed only after storage for two months, when the higher viability was found with the suspension of the older cells. Now Wills (1957) has shown that under otherwise identical conditions the stationary phase of growth of an organism was attained much more rapidly in aerated than in non-aerated media, and although the number of counts from non-aerated media is limited, it would appear that the more stable suspensions may result from the more slowly growing organisms in static, non-aerated media.

Table 7. *The effects of cultural conditions on the viability during storage of washed suspensions of* E. coli

Culture conditions	Age of culture (hr)	Viable count (orgs/ml) after storage for (months)			
		0	1	2	3
Peptone broth, aerated	12	$2{\cdot}50\times10^9$	$3{\cdot}18\times10^6$	$2{\cdot}32\times10^3$	$1{\cdot}02\times10^2$
	16	$2{\cdot}55\times10^9$	$4{\cdot}50\times10^7$	$9{\cdot}78\times10^5$	$1{\cdot}57\times10^6$
	24	$1{\cdot}74\times10^9$	$1{\cdot}06\times10^7$	$8{\cdot}82\times10^6$	$2{\cdot}60\times10^6$
	30	$1{\cdot}50\times10^9$	$8{\cdot}16\times10^7$	$1{\cdot}72\times10^6$	$1{\cdot}24\times10^6$
	36	$1{\cdot}63\times10^9$	$1{\cdot}05\times10^7$	$5{\cdot}46\times10^6$	$7{\cdot}68\times10^6$
	48	$1{\cdot}48\times10^9$	$1{\cdot}36\times10^6$	$3{\cdot}26\times10^5$	$1{\cdot}34\times10^6$
	65	$1{\cdot}39\times10^9$	$1{\cdot}04\times10^8$	$1{\cdot}07\times10^6$	$1{\cdot}00\times10^6$
Peptone broth, non-aerated	24	$1{\cdot}63\times10^9$	—*	$2{\cdot}83\times10^7$	—
	48	$1{\cdot}78\times10^9$	—	$1{\cdot}66\times10^8$	—
Agar slope, moist	24	$1{\cdot}96\times10^9$	$1{\cdot}04\times10^7$	$1{\cdot}21\times10^7$	$7{\cdot}20\times10^6$
Agar slope, dried	24	$1{\cdot}92\times10^9$	$2{\cdot}65\times10^6$	$1{\cdot}56\times10^6$	$9{\cdot}48\times10^5$
Agar plate, moist	24	$1{\cdot}97\times10^9$	$6{\cdot}96\times10^6$	$9{\cdot}12\times10^6$	$2{\cdot}09\times10^6$
Agar plate, dried	24	$1{\cdot}99\times10^9$	$2{\cdot}42\times10^6$	$6{\cdot}23\times10^6$	$7{\cdot}74\times10^5$
Agar plate, nearly full, sealed	24	$2{\cdot}01\times10^9$	$5{\cdot}28\times10^6$	$7{\cdot}86\times10^6$	$6{\cdot}60\times10^6$
Agar plate, sealed over soda lime	24	$1{\cdot}74\times10^9$	—	$2{\cdot}49\times10^8$	—

* $1{\cdot}12\times10^9$ after 7 days.

A difference was detected between the rates of loss of viability of suspensions derived from cells grown on solid and in liquid media. With the former, the viability fell sharply in the first month, with little further decrease during the two succeeding months; with the latter, the rate of viability loss tended to be more even over the three months, although the greatest loss still generally occurred in the first month of storage. Growth on a dried solid medium appeared to give a suspension which retained viability less well than growth from a moist medium.

Discussion

The previously expressed view (Cook, Steel & Wills, 1956) that aqueous suspensions of bacteria might profitably be used in evaluating bactericidal action has been well supported by the results described above. Washed *E. coli* suspended in phosphate buffer (0·1 M, pH 7·0) maintained its viability and bactericide resistance better than did unwashed or washed cells suspended in water; and with such buffered suspensions stored for five weeks at room temperature, variations in phenol resistance were not significantly greater than those likely to be encountered with freshly prepared suspensions.

It has been shown that the rate of loss of bactericide resistance in a stored suspension of *E. coli* is approximately constant for several bactericides. This has obvious application in tests where bactericidal activity is compared with that of a reference substance. Although the limitations of such comparative tests have been adequately pointed out, notably by Berry (1951) and Wilson & Miles (1946), and the defects in the designs of such tests indicated by Cook & Wills (1956*a*), the great majority of disinfectants are still evaluated by a phenol coefficient method. The standard method, even with its rigid specifications for maintenance of cultures of the test organism, is known not to give consistent results between different workers in different laboratories, and it is suggested that the use of a bacterial suspension distributed to those who evaluate bactericides may offer a means of achieving greater consistency. Compared with the test organism at present employed, a suspension of *E. coli* offers advantages in retention of resistance on storage and also in being non-pathogenic.

The authors gratefully acknowledge the valuable technical assistance given by Mr. A. Edwards. The present address of B. A. Wills is: Research and Control Division, Allen & Hanburys (Africa) Ltd., Congella Road, Durban, South Africa.

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EXOCELLULAR PRODUCT FORMATION BY MICRO-ORGANISMS IN CONTINUOUS CULTURE. I. PRODUCTION OF 2:3-BUTANEDIOL BY *AEROBACTER AEROGENES* IN A SINGLE STAGE PROCESS

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SUMMARY: A quantitative study of the growth and carbohydrate metabolism of *Aerobacter aerogenes* in continuous culture with particular reference to 2:3-butanediol production is described. The effects of varying dilution rate, oxygen uptake rate, pH value, temperature and CO_2 pressure were studied.

The optimum pH value was 5·0–6·0. The optimum temperature was 35–37° with a dilution rate of 0·2 hr^{-1}. The rate of sugar metabolism was decreased by raising the CO_2 pressure.

In continuous culture the maximum diol output rate/l. of working volume exceeded the greatest rate reported for batch culture by a factor of three. The maximum diol concentration and yield as a percentage of the theoretical maximum were lower in continuous culture than in batch culture. Reasons for this are given.

Some variants of the parent organism were found in continuous cultures but they caused no change in the yield of butanediol. There was no sign of loss of butanediol-producing ability by any of the cultures, some of which were maintained for 2,000 hr. For the first few generations the rate of production of butanediol actually increased.

Our experience shows that a continuous bacterial culture is far easier to control than a batch culture. Its wider application in the laboratory and in industry should therefore be advantageous. So far, the continuous culture technique has been applied mainly to the production of micro-organisms, and the objects of this work were to investigate its value for exocellular product formation and to compare its suitability for this purpose with that of batch culture, as shown by data from the literature.

The process chosen for study was the production by *Aerobacter aerogenes* of 2:3-butanediol, one of the main end products of the carbohydrate metabolism of that organism, a process which takes place under either anaerobic or partially aerobic conditions. Work on the production of 2:3-butanediol by the batch method of fermentation has recently been exhaustively reviewed by Ledingham & Neish (1954). There is fairly good agreement between different authors on the yields of products and output rates in batch cultures, so we have not re-investigated the production of butanediol in batch cultures. A preliminary report of part of this work has been published (Pirt & Callow, 1958*a*).

Methods

Organism and culture purity tests. The organism used was *Aerobacter aerogenes* 8017 from the National Collection of Industrial Bacteria, which strain is the same as B199 of the Northern Regional Research Laboratory, Peoria, Illinois. It was characterized

by the biochemical tests given by Cowan (1956) and its reactions in these tests were checked from time to time. The purity of cultures was checked by plating on tryptic meat digest agar, and by Gram stain and microscopic examination. The stock cultures were maintained on tryptic meat digest agar.

Culture medium. The medium consisted of (amounts in g/l.): $MgSO_4.7H_2O$, 0·3; $CaCl_2.6H_2O$, 0·09; $FeSO_4.7H_2O$, 0·0225; $ZnSO_4.7H_2O$, 0·0075; $MnSO_4.4H_2O$, 0·00375; ethylenediaminetetraacetic acid (EDTA), 0·51; KOH, *c.* 0·45; $(NH_4)_2SO_4$, 7·2; $(NH_4)_2HPO_4$, 6·0; H_3PO_4, *c.* 0·57 ml; sucrose, 100. The medium was sterilized in three parts: (1) the sucrose, (2) the mixed ammonium salts and the phosphoric acid, and (3) the other inorganic salts with the EDTA. The pH value after sterilizing and mixing was about 6·8.

For the control of pH 2N KOH and 0·5N H_2SO_4 were used. The acid was not normally needed, and the volume of 2N KOH required was about 5% of the medium volume.

Antifoams. The antifoams used were: Alkaterge-C (Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A.) diluted to 30% v/v with liquid paraffin; Antifoam 60 (a silicone emulsion supplied by the General Electric Company, New York) diluted to 30% v/v with water; silicone Antifoam A diluted to 75% w/v with silicone fluid MS.200/10 C.S. (Midland Silicones, 19, Upper Brook Street, London, W.1). Some observations made on foam control in this work are reported elsewhere (Pirt & Callow, 1958*b*).

Inoculum. The continuous culture was inoculated with about 100 ml of culture grown in a shake flask with an oxygen solution rate, measured by sulphite oxidation, of about 45 m-mole/l./hr. To prevent lag in the development of the seed culture the bacteria were first inoculated into a medium in which the concentration of minerals was decreased to one-fifth and the sucrose concentration was 5 g/l. After good growth had occurred, a portion of the culture was transferred to a medium in which the mineral strength was increased to the maximum and the sucrose concentration was 25 g/l. This culture was used to inoculate the continuous culture.

Continuous culture apparatus and technique. Continuous cultures were carried out in the 2 l. scale apparatus described by Elsworth, Meakin, Pirt & Capell (1956). For the control of pH value the automatic control method of Callow & Pirt (1956) was used. The volume of alkali added was measured and the medium flow rate was corrected accordingly. A sufficient time to allow for the culture to reach the steady state was generally four times the replacement time (defined below).

Aeration. The oxygen uptake rate of the culture is given the symbol σ_c and the oxygen solution rate determined by sulphite oxidation (Cooper, Fernstrom & Miller, 1944) is given the symbol σ_s, the units being m-mole O_2/l./hr. The conditions for obtaining the oxygen solution rates have been previously described (Elsworth *et al.* 1956). The rate of stirring was 1000 rev/min, unless σ_s values less than 50 m-moles/l./hr were required, in which case a lower stirring rate was used; other exceptions are noted. The relationship between σ_c and σ_s is considered elsewhere by Pirt & Callow (1958*c*).

Analytical methods. To preserve the culture sample, saturated $HgCl_2$ was added to the sample immediately after it was taken (*c.* 0·05 ml/50 ml sample). The bacteria

were then spun down and the supernatant was stored at 3°. The methods of determining bacterial dry weight, acetoin, ethanol and reducing sugar have been previously described (Pirt, 1957). Lactic acid was estimated by the method of Barker & Summerson (1941). Formic acid was determined by the method of Grant (1947).

The amount of cell carbon was estimated by multiplying the dry weight by the factor 0·485. This factor was determined by making some carbon analyses by combustion of the dried bacteria.

2:3-butanediol was determined by oxidation to acetaldehyde by periodic acid and colorimetric determination of the acetaldehyde (Neish, 1952; Desnuelle & Naudet, 1945). Applied directly to diluted samples of culture fluid the method gave good agreement with estimations by the more time-consuming method previously described by Pirt (1957). A correction was made for the acetoin present, which produced half as much colour intensity/mole as did butanediol.

The method of determining sucrose was based on that of Freeman & Morrison (1946). Sucrose, adjusted to a concentration of 0–5 mg/ml, was hydrolyzed with 0·5N HCl at 70° for 10 min. After neutralization with NaOH and then making just acid to phenolphthalein with HCl the reducing sugar was estimated.

Oxygen and carbon dioxide in the effluent gas of the culture were determined by means of an Orsat apparatus. The gas analysis technique was basically that described by Strouts, Gilfillan & Wilson (1955). The gas flow rate was metered on the outlet side by a Rotameter (Rotameter Manufacturing Co., Croydon). Estimation of the total volume of gas produced by the culture (by gas flow rate measurements on both the inlet and outlet gas streams), combined with CO_2 and O_2 analyses, showed that the gas volume change was entirely accounted for by the O_2 consumption and CO_2 production. Hence the volume of O_2 consumed (V_o) may be calculated by means of the formula $V_o = \frac{V_e}{100}\left[\frac{20{\cdot}9\{100-(a+b)\}}{79} - b\right]$, where V_e is the volume of gas leaving the culture vessel, and a and b are the percentages of CO_2 and O_2 in the effluent gas respectively. The amount of CO_2 in the inlet air can be neglected. Retention of CO_2 was corrected for at pH values above 6·0 by application of the Henderson-Hasselbach equation (Umbreit, Burris & Stauffer, 1948). In this calculation it was assumed that the dissolved CO_2 was in equilibrium with the CO_2 in the effluent gas. Previous work (Pirt, 1957) had shown that this assumption was substantially correct.

Replicates. Two types of replicate results are possible. Culture conditions may be reproduced either in different cultures or at different times in the same culture. Two or more replicate results were obtained in one of these ways for each of the culture conditions investigated, and only the mean values have been used. The variation in the results is considered below, and standard deviations are given (Tables 4 and 5).

Symbols, units and calculations. The terminology for the description of culture conditions and behaviour is rapidly increasing and unfortunately some of the symbols have more than one meaning attributed to them. The definitions of the symbols and units of quantity we use are as follows: x is bacterial dry weight concentration (g/l.); t is time (hr); $\frac{dx}{dt}$ is culture growth rate (g/l./hr); $\mu\left(=\frac{1}{x}\cdot\frac{dx}{dt}\right)$ is specific growth

rate (hr^{-1}), μ_m being the maximum value of μ; t_d is doubling time, the time for the bacterial dry weight to double itself (hr); $D\left(=\frac{F}{V}\right)$ is the dilution rate (hr^{-1}), F (l./hr) being the medium flow rate and V. (l.) the culture volume, so that D is measured in hr^{-1} and $D=\mu \frac{\ln 2}{t_d}$ (Herbert, Elsworth & Telling, 1956): $t_r\left(=\frac{1}{D}\right)$ is the replacement time (hr); $Y\left(=\frac{\Delta C}{X}\right)$ is the cell yield constant, where ΔC is the amount of carbon substrate utilized to produce X g dry weight of bacteria; and q is a metabolic quotient (g of substance utilized or produced/hr/g dry weight of bacteria), though in the case of oxygen, for greater convenience, the units are m-moles O_2/hr/g dry weight. The substance concerned is indicated by a suitable subscript, e.g. q_{o_2} for oxygen, q_c for carbon substrate. The most direct way of calculating q from continuous culture results is by means of the formula $q=\frac{D(\Delta S)}{x}$ where ΔS (g/l.) is the difference, considered positive, between the amount of substance supplied and the amount remaining in the culture. The $q_{o_2}=\frac{\sigma_c}{x}$.

Results

The selection of the medium

A defined medium to give a bacterial concentration not less than 10 mg dry wt/ml and a high growth rate was required. The medium given in the methods was found to meet these requirements. The growth curve is given in Fig. 1. Initially μ_m was 0·5. The point A at which μ_m changed abruptly from 0·5 to 0·3 corresponded closely to the point at which, it was calculated, oxygen supply would become a limiting factor. A bacterial concentration of 12 mg dry wt/ml was obtained under the growth conditions stated in Fig. 1. The maximum diol concentration reached in batch culture was 15 g/l. but no attempt was made to find the conditions for maximum production.

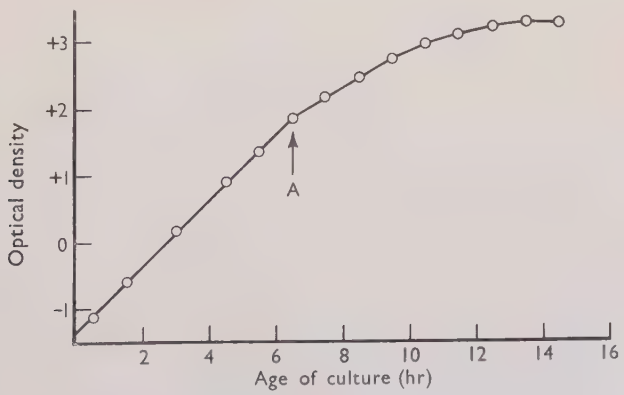

Fig. 1. The growth curve of *Aerobacter aerogenes* in batch culture. Oxygen solution rate (σ_s), 130 m-moles/l./hr; temperature, 35°; pH 5·5. At point A the specific growth rate (μ_m) changed from 0·5 to 0·3 hr^{-1}.

The effects of varying the oxygen supply and the dilution rate

It has previously been shown (Ledingham & Neish, 1954; Freeman, 1947) that aeration of an *A. aerogenes* culture suppresses ethanol and lactic acid formation and increases the yield of butanediol; too great an aeration increases the acetoin production at the expense of diol production. Pirt (1957) showed that butanediol is produced in anaerobic or partially aerobic metabolism. The latter occurs when respiration is limited by the oxygen supply. The aeration in previous work was measured in terms of rate of air flow through the culture. This is inadequate because it does not express the amount of available oxygen. In this paper the oxygen uptake rate of the culture is given for each set of conditions, and quantitative relationships between the oxygen supply and the amounts of products formed have been found. The oxygen requirement of a growing culture depends on the specific growth rate or dilution rate in continuous culture, and the bacterial concentration (Pirt, 1957).

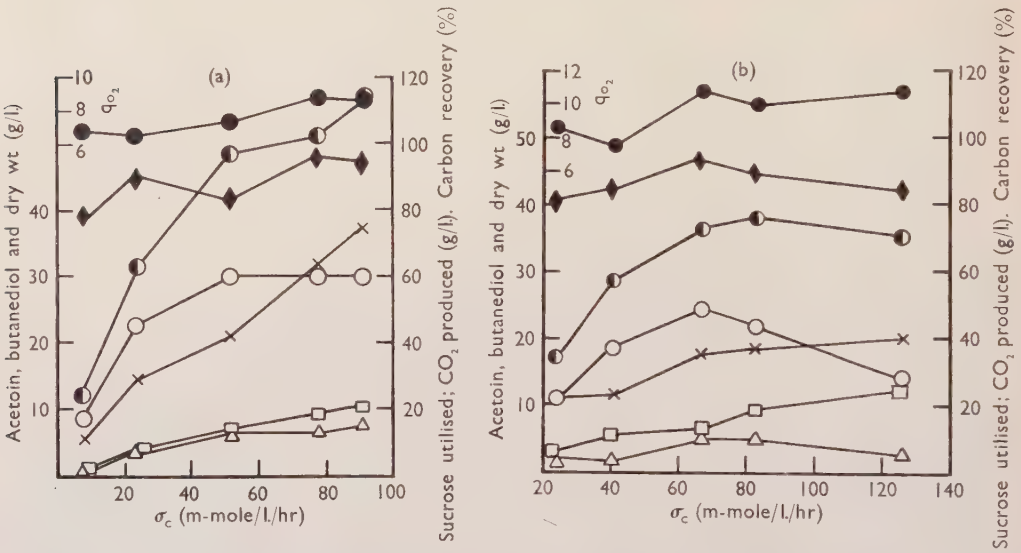

Fig. 2. Amounts of metabolic products as a function of rate of oxygen uptake (σ_c). (a) Dilution rate, 0·11 hr^{-1}; culture L. (b) Dilution rate, 0·20 hr^{-1}; cultures A, B, C and L. Temperature, 35°; pH, 5·5. Full circles, q_{O_2} (m-mole/hr/g dry wt.); half-full circles, sucrose utilized; full diamonds, percentage of carbon recovered as diol, acetoin, CO_2 and bacteria; open circles, 2:3-butanediol; open squares, bacterial dry wt.; open triangles, acetoin; crosses, CO_2 produced.

Table 1. *The amounts of metabolic products formed at different dilution rates, with the same oxygen supply*

Dilution rate (hr^{-1})	Sucrose utilized (g/l.)	Bacterial dry wt (g/l.)	Butane-diol (g/l.)	Acetoin (g/l.)	CO_2 (g/l.)	Carbon recovery (%)	q_{O_2} (m-moles O_2 /hr/g/dry wt)	q_c (g sucrose utilized /hr/g/dry wt)
0·11	114	10·6	30·0	7·5	74·5	95·0	8·6	1·11
0·20	75	9·5	20·0	4·2	38·5	88·9	9·6	1·58
0·32	42	7·5	12·5	2·0	20·0	95·3	12·1	1·79

Rate of oxygen uptake (σ_c), 91 m-moles/l./hr; temperature, 35°; pH 5·5; cultures A and L.

Effects on growth. The effects of varying the oxygen supply at two different dilution rates are shown in Fig. 2, and the effect of varying dilution rate with a fixed oxygen uptake rate is shown in Table 1. All nutrients other than oxygen were in excess. To ensure this with D=0·1 the sucrose concentration was raised to 14% w/v. From the effects observed the following approximate quantitative relationships may be derived. When oxygen uptake rate (σ_c) was variable, the bacterial concentration (x) was directly proportional to σ_c. The relationships were: $x=0{\cdot}12\sigma_c$ with D=0·10 and $x=0{\cdot}10\sigma_c$ with D=0·20.

From these results the yields of bacteria/m-mole of O_2 in partially aerobic metabolism can be calculated; they are given by the expression Dx/σ_c. The yields in g dry wt/m-mole O_2 were: 0·013 with D=0·11, 0·020 with D=0·20 and 0·024 with D=0·30. Hence the yield increased with the specific growth rate.

Effects on sucrose utilization. At low σ_c values the amount of sucrose utilized (ΔC) was directly proportional to σ_c. When dilution rate was the variable and σ_c was constant, $D\Delta C$ was approximately constant.

The effect of σ_c on the sucrose metabolic quotient (q_c) is shown in Fig. 3. In general, q_c increased with decrease in σ_c, which is the Pasteur effect.

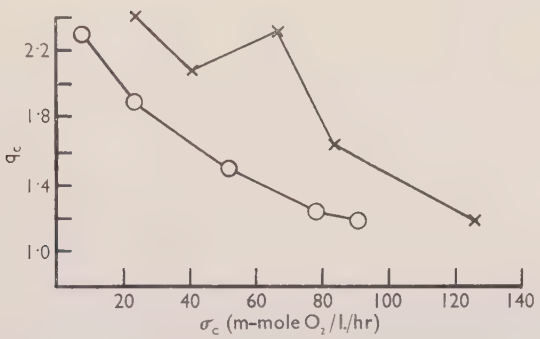

Fig. 3. Sucrose metabolic quotient (q_c, g sucrose utilized/hr/g dry wt.) as a function of rate of oxygen uptake (σ_c). Open circles, dilution rate (D), 0·11 hr^{-1}; temperature, 35°; pH, 5·5; culture L. Crosses, D, 0·20 hr^{-1}; temperature, 35°; pH, 5·5; cultures A, B, C and L.

The sucrose metabolic quotient was shown to be independent of culture sucrose concentration in the range 4–40 g/l., that is, the range within which practically all the steady state sucrose concentrations fell.

Effects on CO_2 production and q_{O_2}. At low σ_c values, <50 with D=0·10 and <70 with D=0·20, about 30% of the utilized substrate carbon was converted to CO_2. At higher σ_c values the percentage conversion of carbon substrate to CO_2 was higher. When D was the variable and σ_c was constant, D multiplied by the amount of CO_2 produced was approximately constant.

Changes in σ_c, other things being equal, had little effect on the q_{O_2} (Fig. 2). For instance, with D=0·1, when σ_c increased twelvefold the q_{O_2} only increased from 6·5 to 8·5. Change in the oxygen supply at a given growth rate was taken up, principally, by change in the bacterial concentration. Increase in specific growth rate or dilution rate caused some increase in the q_{O_2} (Table 1).

Effects on butanediol and acetoin production. The maximum diol concentration with D=0·10 was about 30 g/l.; it occurred when σ_c was 50 or more. With D=0·20 the maximum diol concentration was about 23 g/l. and occurred with σ_c=67. With D=0·30 the maximum diol concentration, 14 g/l., occurred with σ_c=67. The diol yield as a percentage of the theoretical maximum (1 mole diol/mole of hexose) is given in Fig. 4, together with the ratio moles diol:moles acetoin. The maximum percentage yield of diol (68%) was obtained with D=0·11 and σ_c=23·5. The maximum percentage yield obtained with D=0·20 was 59%. The diol:acetoin ratio increased sharply at very low σ_c values with D=0·10. In batch cultures under optimum conditions, Freeman (1947) found the diol:acetoin ratio about 20:1, that is, about equal to what we found with the lowest oxygen supply rate (σ_c=8).

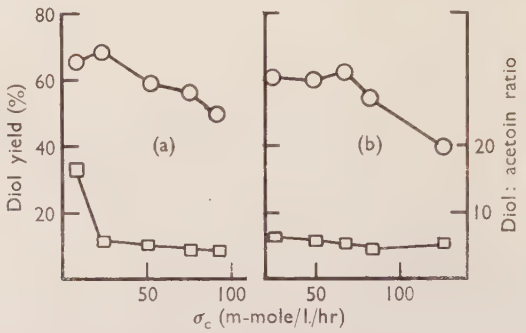

Fig. 4. Diol yield, as percentage of the theoretical maximum, and the ratio, diol: acetoin, as functions of oxygen uptake rate (σ_c). Temperature, 35°; pH, 5·5. (a) Dilution rate, 0·11 hr^{-1}; culture L. (b) Dilution rate, 0·20 hr^{-1}; cultures A, B, C and L. Open circles, percentage diol yield; open squares, ratio, moles diol:moles acetoin.

Production of ethanol, lactic acid and formic acid. Only traces of ethanol, lactic acid and formic acid were produced in partially aerobic metabolism at pH values below 6, e.g., at pH 5·5 with D=0·10 and σ_c=52, ethanol, lactic acid and formic acid respectively accounted for 1·5%, 0·5% and 0·1% of the total carbon. With the smallest oxygen uptake rate (σ_c=8 and D=0·1) the amounts were appreciably greater: ethanol contained 4·0%, lactic acid 1·9% and formic acid 2·5% of the total carbon utilized. However, at higher pH values much more lactic acid was found. Even with the lowest oxygen uptake rate the metabolism differed fundamentally from anaerobic metabolism. For comparison some study of anaerobic metabolism was made.

Anaerobic metabolism. When nitrogen instead of air was supplied to the culture at pH values of 6 and below, the bacterial concentrations produced were so small (0·3 mg/ml or less) that it was doubtful whether a true steady state existed. There was some growth on the walls of the vessel, and at such a low cell density this may have appreciably affected the results. The only results which could be considered reliable with the anaerobic conditions were those obtained at pH 7·0. They are given in Table 2. Partially aerobic metabolism at pH 7·0 is considered below. Depriving the culture of oxygen caused a large increase in the percentage conversion

of sucrose to lactic acid and considerable increases in the production of formic acid and ethanol; it caused a decrease in the percentage conversion of substrate to diol.

Table 2. *The products formed during the anaerobic metabolism of sucrose by* Aerobacter aerogenes *in continuous culture*

pH	Sucrose utilized (g/l.)	Bacterial dry wt (g/l.)	Butane-diol (g/l.)	Acetoin (g/l.)	Lactic acid (g/l.)	Formic acid (g/l.)	Eth-anol (g/l.)	CO_2 (g/l.)	Carbon recovery (%)	q_c (g sucrose utilized /hr/g dry wt)
6·0	17·5	0·3	1·5	0	6·9	1·0	1·5	2·35	73	5·8
7·0	25·6	0·92	4·06	0	8·10	2·67	2·40	4·63	87	2·78

Dilution rate, 0·10 hr^{-1}; temperature, 35°; culture L.

The effects of pH value changes

In the butanediol fermentation pH value has generally been controlled by the addition of $CaCO_3$. This method we replaced by the automatic pH control system. As a result we were able to vary the pH value at will and control it within 0·05 units for periods of hundreds of hours. Previous work with batch cultures has shown that the optimum pH value for butanediol production lies within the range 5·0–6·2

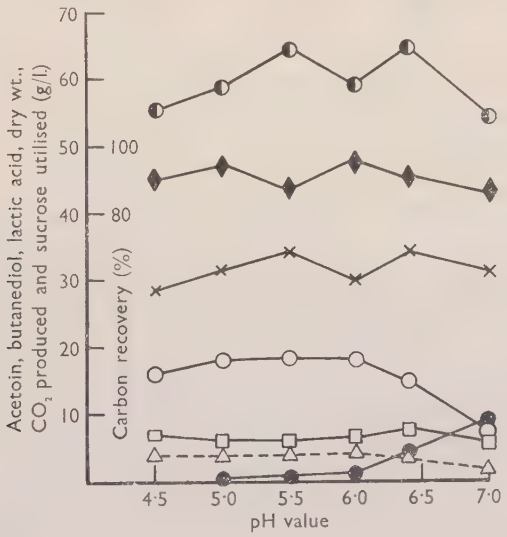

Fig. 5. Amounts of metabolic products as a function of pH value. Dilution rate, 0·20 hr^{-1}; rate of oxygen uptake (σ_c), 55 m-moles/l./hr; temperature, 35°; culture L. Half-full circles, sucrose utilized; full diamonds, carbon recovery (including formic acid and ethanol); crosses, CO_2; open circles, diol; open squares, bacterial dry wt.; open triangles, acetoin; full circles, lactic acid.

(Ledingham & Neish, 1954; Freeman, 1947). The effect of pH value on the anaerobic metabolism of *A. aerogenes* and other butanediol producing bacteria has been determined by Neish & Ledingham (1949). The effect of pH value on the partially aerobic metabolism of *A. aerogenes* in continuous culture is shown in Fig. 5. Maximum diol production occurred in the pH range 5·0–6·0. At pH values above 6·0 the diol pro-

duction decreased in favour of lactic acid production, and at pH 7·0 the concentration of lactic acid was 8·9 g/l. Freeman (1947) also found an increase in lactic acid production with increase in pH value. Formic acid was found only in traces (0·1% of the total carbon at pH 5·0 and 1·5% at pH 7·0). Ethanol production accounted for 2% of the total carbon at pH 5·0 and 3% at pH 7·0. Acetic acid could not be detected in the volatile acid fraction by the sensitive lanthanum nitrate test. The effect of pH value on diol yield as a percentage of the theoretical maximum and on the diol:acetoin ratio is shown in Fig. 6. The percentage yield of diol was at a maximum in the pH range 4·5–6·0; at higher pH values it decreased rapidly. The diol:acetoin ratio was unaffected by pH value.

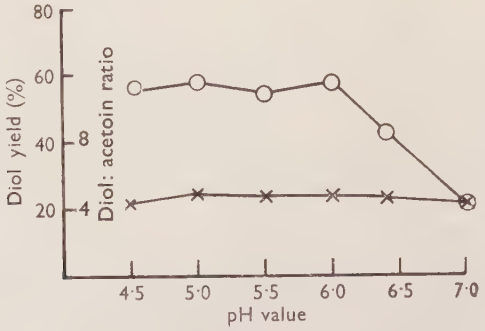

Fig. 6. The effect of pH value on the yield of diol (as percentage of theoretical maximum, open circles) and on the diol:acetoin ratio (crosses), in culture L.

The effect of pH value on the sucrose metabolic quotient is shown in Fig. 7a. The bacteria utilized sucrose most rapidly at pH 5·5. Hence at that value maximum yield of diol combined with fastest conversion of sucrose occurred.

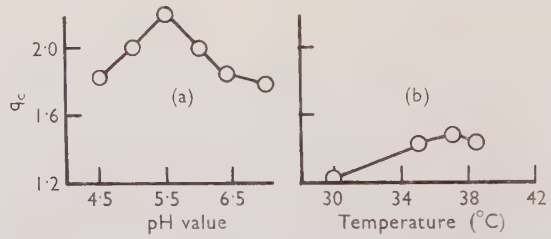

Fig. 7. The effects of pH value (a) and temperature (b) on the sucrose metabolic quotient (q_c, g sucrose utilized/hr/g dry wt.). Conditions for (a): dilution rate, 0·20 hr^{-1}; rate of oxygen uptake (σ_c), 55 m-mole/l./hr; temperature, 35°; culture L. (b) Dilution rate, 0·20 hr^{-1}; σ_c, 91 m-mole/l./hr; pH, 5·0; cultures A and C.

The accurate automatic control of pH value revealed that the organism took a considerable time to adapt to downward changes, at least at values below 6·0. A sudden drop of 0·5 units stopped growth for several hours. We therefore adopted the practice of making pH changes at the rate of 0·1 units/hr.

The effects of temperature changes

The effects of temperature changes in the range 30–37° are depicted in Fig. 8. Above 37° the growth was very sensitive to temperature changes and at 40° no steady state was obtained, the growth rate falling practically to zero. At lower temperatures the yield of bacteria (Y) increased slightly and a smaller proportion of the substrate carbon was converted into CO_2. With D=0·2 the optimum temperature for diol production was 35–37°. The effect of temperature on the sucrose metabolic quotient is shown in Fig. 7b. Similar results were obtained with D=0·1, except that the optimum temperature for diol production was probably 30–35°. Unfortunately the

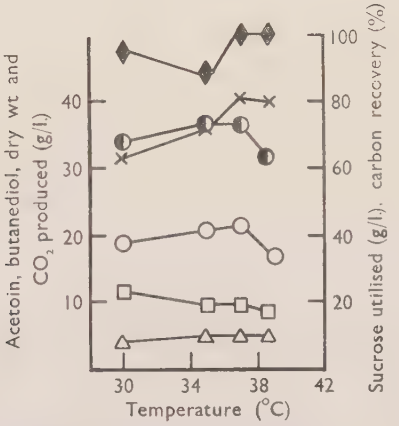

Fig. 8. Amounts of metabolic products as a function of temperature. Dilution rate, 0·20 hr^{-1}; rate of oxygen uptake (σ_c), 91 m-mole/l./hr; pH, 5·0; cultures A and C. Full diamonds, percentage carbon recovered as diol, acetoin, bacteria and CO_2; crosses, CO_2; half-full circles, sucrose utilized; open circles, diol; open squares, bacterial dry wt.; open triangles, acetoin.

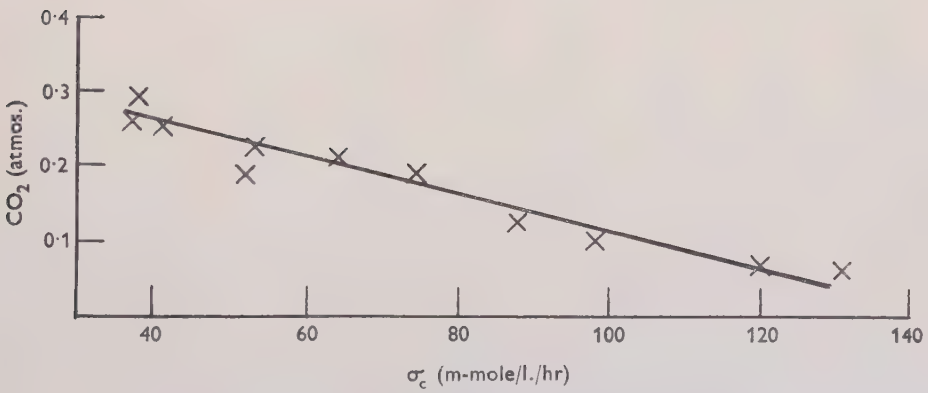

Fig. 9. The partial pressure of CO_2 in the effluent gas as a function of rate of oxygen uptake (σ_c) with stirring at 1000 rev/min. Dilution rates in the range 0·1–0·2 hr^{-1}; temperature 35°–37°; pH, 5·0–5·5; cultures A, C and F.

results with D=0·1 were not conclusive because the sugar supplied (100 g/l.) was not sufficient to maintain an excess at all temperatures. It is interesting to note that the optimum temperature may be a function of the dilution rate.

The effect of CO_2 pressure changes

The CO_2 pressure varied, due to the varying rate of CO_2 output by the culture and the different air flow rates necessary to produce the required rate of oxygen uptake. With stirring at 1000 rev/min there was a linear relationship between the partial pressure of CO_2 and σ_c, as shown in Fig. 9. It has been suggested that the CO_2 pressure might affect the metabolism (Ledingham & Neish, 1954), and some experiments were carried out to see if this were so. The CO_2 pressure was varied by

Table 3. *The effect of CO_2 pressure on the partially aerobic metabolism of* Aerobacter aerogenes (*culture* L)

CO_2 partial pressure (atmos)	Rate of stirring (rev/min)	Bacterial dry wt (g/l.)	Sucrose utilized (g/l.)	Butane-diol (g/l.)	Acetoin (g/l.)	CO_2 (g/l.)	q_c (g sucrose utliized /hr/g dry wt)
Dilution rate, 0·21 hr^{-1}; oxygen uptake rate (σ_c), 55 m-mole/l./hr; temp., 35°; pH, 5·5							
0·097	700	6·2	65·8	20·5	3·8	35·3	2·23
0·256	1000	6·0	66·2	18·4	4·5	33·8	2·31
Dilution rate, 0·21 hr^{-1}; σ_c, 41 m-moles/l./hr; temperature, 35°; pH, 5·1							
0·026	600	5·0	62·0	20·7	3·3	32·6	2·60
0·311	1000	4·8	51·8	16·7	3·0	28·6	2·26

Table 4. *The variation in results with increase in number of generations (or culture age)*

Age of culture† (hr)	Sucrose utilized (g/l.)	Bacterial dry wt (g/l.)	CO_2 (g/l.)	Butane-diol (g/l.)	Acetoin (g/l.)	Carbon recovery (%)	q_c (g sucrose utilized /hr/g dry wt)
Culture F; dilution rate, 0·19 hr^{-1}; oxygen uptake rate (σ_c), 89 m-moles/l./hr; temperature, 37°; pH, 5·0							
48	59·0	9·8	33·1	7·4	3·0	77	1·14
97	61·0	9·4	36·7	14·0	4·5	94	1·23
193	73·0	9·8	37·4	16·0	5·0	85	1·41
289	74·0	9·2	38·0	15·0	5·3	83	1·53
433	72·5	9·4	41·0	17·4	5·5	91	1·48
457	77·5	9·0	36·1	16·3	5·6	80	1·54
794	74·5	10·8	36·8	17·3	4·0	86	1·31
818	71·5	9·2	35·3	17·0	4·0	85	1·48
Mean*	73·8	9·6	37·4	16·5	4·9	85	1·46
Standard deviation*	2·1	0·66	2·0	0·9	0·73	3·6	0·09

† 100 hr corresponds to 27·4 doubling times or generations.
* Of results after 97 hr, i.e. after q_c had reached the maximum.

changing the air flow rate while σ_c was kept constant by changing the rate of stirring. The results, given in Table 3, show that on changing the CO_2 partial pressure from 0·097 atmospheres to 0·256 atmospheres there was no significant change in the metabolism. Practically all our results were obtained with CO_2 pressures in this range. Comparison of the metabolism at CO_2 partial pressures of 0·026 atmospheres

and 0·311 atmospheres indicated that the sucrose metabolic quotient was appreciably higher at the lower CO_2 pressure and consequently more sucrose was converted.

Variation in the results

Effect of number of generations. At the beginning of a culture we found that the sucrose metabolic quotient increased for many generations with constant environmental conditions. We followed this effect in one culture (Table 4). Its magnitude varied from culture to culture but generally q_c initially was 10–30% less than the maximum attained and took between 10 and 50 generations to reach the maximum value. The bacterial concentration did not vary significantly with increase in the number of generations. Therefore, as a consequence of the increase in q_c, the amount of sucrose utilized/l. and the concentration of diol were increased. This phenomenon can complicate the comparison of results, and to avoid this all comparisons of effects were made after q_c had reached the maximum.

Variation between cultures. The extent of variation between cultures is represented in Table 5. The cultures were run in the order A, B, C, etc. The first three cultures gave higher diol concentrations than the later ones: this variation remains unexplained. On the whole, the variation between cultures was not significantly different from the variation between replicates in the same culture.

Table 5. *The variation between cultures**

Culture	Sucrose utilized (g/l.)	Dry wt (g/l.)	CO_2 (g/l.)	Butanediol (g/l.)	Acetoin (g/l.)	Carbon recovery (%)	q_c (g sucrose utilized /hr/g dry wt)
Dilution rate, 0·10 hr^{-1}; oxygen uptake rate (σ_c), 44 m-moles/l./hr.; temp., 30°; pH, 5·0							
A	89·5	8·2	41·5	34·3	5·7	97	1·09
B	92·5	8·1	43·1	31·6	6·2	92	1·14
C	98·3	7·8	43·5	27·1	6·4	90	1·26
D	96·5	9·0	46·3	26·2	6·9	87	1·07
E	91·0	7·9	43·4	28·3	6·4	90	1·15
M	85·1	8·5	46·1	27·6	3·3	93	1·00
Mean	92·2	8·3	44·0	29·2	5·8	91·5	1·12
Standard deviation	4·8	0·44	1·86	3·2	1·3	3·4	0·088
Dilution rate, 0·19 hr^{-1}; σ_c, 89 m-moles/l./hr; temperature, 37°; pH, 5·0							
A	73·9	10·0	40·0	21·4	5·0	99	1·40
C	68·5	9·4	35·2	21·0	3·5	102	1·25
E	75·6	9·3	34·8	19·8	5·3	85	1·54
F	74·0	9·3	36·9	17·0	4·8	84	1·51
M	70·5	10·3	35·8	17·1	3·7	87	1·30
Mean	72·5	9·7	36·5	19·3	4·5	91·4	1·40
Standard deviation	2·9	0·47	2·1	2·1	0·81	8·4	0·13
Dilution rate, 0·20 hr^{-1}; σ_c, 66 m-moles/l./hr; temperature, 35°; pH, 5·5							
C	72·8	6·3	35·3	24·7	4·8	92	2·31
L	77·8	6·8	36·3	22·4	4·4	82	2·29
M	69·9	6·5	33·8	22·0	4·0	90	2·15
Mean	73·5	6·53	35·1	23·0	4·4	88	2·25
Standard deviation	4·0	0·26	1·3	1·5	0·40	5·3	0·09

* The results given were all obtained after q_c had reached the maximum.

The presence of variants in continuous cultures

Two variants were found in some of the cultures after they had been run for *c.* 1000 hr. One was a mucoid type, that is, it produced an exocellular slime, identified as a polysaccharide, in plate cultures; colonies of this variant could also be identified by their buff colour, those of the original strain being grey. The other variant was a small colony type. Freeman (1947) reported the isolation of a small colony type from a similar culture of *A. aerogenes*. These variants reproduced true to type. Both were indistinguishable from the original strain by the biochemical tests given by Cowan (1956), all of which were applied with the exception of those for the utilization of malonate and phenylalanine as carbon sources, and for the production of glutamic acid decarboxylase.

The yields and concentrations of products were unaffected by the presence of these variants, even though in one culture (L) their number reached 20% of the bacteria present.

Butanediol recovery and characterization

The butanediol in 2 l. of harvested culture was recovered by the method of Freeman & Morrison (1947). The total amount of diol in the broth was 77·0 g, of which 68% (52·1 g of pure diol) was recovered, 20% (15·5 g) being accounted for in the fractions discarded. The boiling point of the recovered diol was 175–177°; redistilled commercial 2:3-butanediol had a boiling point of 176–177°. The specific rotation of the recovered diol (α_D, 10% w/v solution) was +1·00°.

Discussion

Partially aerobic metabolism

It is well known that anaerobic metabolism in micro-organisms is responsible for the degradation of carbon sources to such major end products as alcohols, ketones and organic acids. About the products of aerobic metabolism, however, the literature is not so definite. This may be attributed to the lack of knowledge about the aeration requirement of a culture. Recently Pirt (1957) showed that aerobic metabolism may be differentiated into fully aerobic and partially aerobic types. Fully aerobic metabolism occurs when there is an excess of oxygen available. Partially aerobic metabolism occurs when respiration is limited by the oxygen supply. Fully aerobic metabolism in an *Aerobacter* species gave only bacteria and CO_2 in large amounts. This paper shows that partially aerobic metabolism can produce large amounts of typical fermentation products such as butanediol and lactic acid. There is not simply one carbon balance for a fermentation, such as seems to be suggested by previous work on the anaerobic type (e.g. see Oginsky & Umbreit, 1954); the carbon balance, we have shown, is a complex function of such variables as oxygen supply, pH value and growth rate. The effect of pH value on the butanediol fermentation roughly accords with Gale's (1951) observation that at acid pH values bacteria tend to produce alkaline or neutral products and at alkaline pH values they form acid products. The effects of growth and pH value seem generally to have been ignored.

In effect we have defined the influence of oxygen on the process in terms of the

oxygen solution rate and the oxygen demand, which is a function of growth rate and amount of available substrate (Pirt, 1957). Probably the effect of oxygen could be more simply and directly determined in terms of the dissolved oxygen concentration. This would require means for the measurement and automatic control of dissolved oxygen concentration over long periods, for which suitable techniques have not yet been developed.

Production of acetoin

One of the problems in butanediol production is to minimize acetoin production. With a given organism this can only be done by keeping the oxygen supply as small as possible. The ratio diol:acetoin also seemed to depend on the type of organism, since we found that *Pseudomonas hydrophila* (strain F14, supplied by the Canadian Research Council, Saskatoon) in continuous culture gave very similar results to *A. aerogenes* except that the ratio diol:acetoin was much lower with the pseudomonad, 2:1 compared with the 5:1 given by *A. aerogenes* under the same conditions.

The detection of acetoin is the basis of the Voges-Proskauer test which is used in the identification of Enterobacteriaceae. In this connection it is important to notice that a small supply of oxygen was necessary for acetoin to be formed; with no oxygen available acetoin formation was completely suppressed. The Voges-Proskauer test would therefore probably be much less frequently ambiguous if cultures were grown under conditions which permit slight aeration, e.g. with a small volume of medium and a large volume of air in the test tube.

Yield, product concentration and rate of product formation

The yield of product from a given amount of substrate, the product concentration, and the output rate/l. of working volume are the important factors in the economics of the process. The concentration of product (e.g. butanediol) is given by the relationship: diol concn. $= \frac{x\,(q_{diol})}{D}$, from which it follows that to obtain the maximum diol concentration, D should be made as small as possible and x and q_{diol} as large as possible. The output rate/l. of working volume is given by the expression: D (diol. concn.) $= x\,(q_{diol})$. A comparison of the various quantities in these equations under different conditions is given in Table 6. Clearly, the maximum values of diol concentration, output rate and percentage yield did not occur under the same conditions. The conditions required for growth and the conditions required for maximum output rate, diol concentration and yield were actually in conflict, for with D=0·11 decrease in σ_c increased the q_{diol} and percentage yield but decreased the amount of organism present.

This analysis of factors which affect product concentration may be applied to batch fermentations. The only change for batch fermentations is that for the term 1/D (the replacement time) one would substitute duration of the fermentation. From the equation it follows that instead of selecting organisms for overall increase in product concentration throughout a fermentation, as is done, for example, in the antibiotics industry, it may be quicker and preferable to select for rate of production/g of organism, that is, $q_{product.}$

Table 6. *A comparison of concentrations, output rates and percentage yields of diol under some critical conditions**

Dilution rate (D) (hr^{-1}) and conditions*	Bacterial dry wt (g/l.)	q_{diol} (g diol/ hr/g dry wt)	Diol (g/l.)	Diol output rate (g/hr/l. of working volume)	Diol yield (% of theoretical maximum)
At maximum diol concentration					
0·11 (a)	7·1	0·44	29·8	3·28	61
0·20 (b)	6·3	0·73	23·0	4·60	59
0·30 (c)	6·0	0·69	13·7	4·10	59
At maximum percentage yield of diol					
0·11 (d)	3·7	0·67	22·5	2·47	68

* Conditions.

	Temp. (°C)	pH	σ_c (m-mole/l./hr)
(a)	35	5·5	52
(b)	35	5·5	67
(c)	37	5·0	67
(d)	35	5·5	23

Comparison of batch and continuous cultures

In Table 7 a comparison of the production of 2:3-butanediol by batch and continuous processes is given. In the batch process, sucrose seems to have been more rapidly converted to butanediol than glucose was. Table 7 shows that the maximum rate of diol production in the continuous process was about three times greater than the maximum rate reported for batch culture, making no allowance for 'turn around' time between batches, which would make the latter even slower relative to the continuous process. The main reason for the difference between the rates of the continuous and batch processes is that in the latter the culture must be grown from a small inoculum, possibly with a lag period, whereas in continuous culture the bacterial concentration is maintained at the maximum all the time.

Table 7. *A comparison of batch and continuous processes for 2:3-butanediol production by* Aerobacter aerogenes

Reference	Carbon source	Diol (g/l.)	Yield (% of theoretical maximum)	Output rate of diol (g/hr/l. of working volume)
Batch fermentation				
Freeman & Morrison (1947)	sucrose	65	87	1·6
Dudykina (1949)	sucrose	58	77	1·6
Ward *et al.* (1945)	hydrolysed starch	42	82	1·1
Freeman & Morrison (1947)	molasses	39	72	0·9
Perlman (1944)	wood hydrolysate	59	70	0·8
Batch fermentation with slow glucose feed				
Olson & Johnson (1948)	glucose	98	74	0·9
Continuous fermentation				
	sucrose A	23	59	4·6
	sucrose B	30	61	3·0

A, Dilution rate 0·20 hr^{-1}; B, Dilution rate 0·10 hr^{-1}.

The yield and concentration of diol were lower in continuous culture than in batch culture. Two causes of the lower yield and concentration could be seen. One was that in continuous culture growth must be continuous, but the conditions required for sufficient growth and the conditions for maximum diol yield and concentration were not identical. The other was that in continuous culture the bacteria flowed out of the culture vessel while they were still capable of converting substrate into diol, so that their capabilities were not utilized to the full. In contrast, in a batch culture the initial conditions may be made suitable for growth and afterwards the conditions may be adjusted to those optimum for diol production, and the bacteria may be kept in the culture vessel as long as they are capable of converting substrate into diol. In order to maintain the optimum conditions for diol production after growth has ceased and to retain the bacteria in a culture vessel as long as they will convert substrate into diol, a two stage continuous process seems to be necessary, the first for growth of the organism, and the second for butanediol production. Such a two stage continuous culture has been investigated and will be described in a subsequent paper (Pirt & Callow, 1959).

The effect of increase in duration of culture

The continuous culture technique has been criticized on the grounds that there would be loss of function or decrease in production due to mutation or genetic recombination, and also difficulty in maintaining sterility for long periods. Herbert, Elsworth & Telling (1956) have replied to this criticism. We have paid careful attention to these aspects. Our continuous cultures each lasted between 200 and 2000 hr and the combined fermentation time was 11,000 hr, that is, 15 months. We did not find contamination in any of the cultures. The metabolic processes investigated were quite stable and no loss of function or decrease in production resulted from increase in the duration of the culture. In order to check on variation in the organism in a continuous culture we have adopted the practice of reproducing some standard conditions at different times during the culture and using the constancy of the results as an index of variation. This practice revealed that the sucrose metabolic quotient increased with increase in the number of generations.

The increase in the sucrose metabolic quotient (q_c) with increase in the number of generations seems to resemble the increase in the rate of hyaluronidase formation/g of bacteria that Rogers (1957) observed in a continuous culture of a *Staphylococcus*. The increase in the maximum specific growth rate (μ_m) of an *Aerobacter* species reported by Pirt (1957) also seems to be a similar effect. In the latter case, since $\mu = q_c Y$ and Y was almost constant over the range of μ values concerned (0·46–0·70), it follows that there was an increase in q_c. The increase in q_c may have been due either to an adaptive mechanism in the cell or to mutation and selection. Which of these mechanisms was responsible it is not possible to decide with the present data.

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the analyses. Many thanks too are due to the team who have kept long and careful supervision of the apparatus.

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THE RELATIONSHIP BETWEEN MAXIMUM OXYGEN SOLUTION RATES IN A BACTERIAL CULTURE AND IN A SODIUM SULPHITE SOLUTION UNDER COMPARABLE AERATION CONDITIONS

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SUMMARY: The maximum possible oxygen solution rates in bacterial cultures with different aeration conditions were appreciably less (0·5–0·7 times) than those in a sodium sulphite solution containing a copper catalyst under the same aeration conditions. Knowledge of the relationship between the maximum oxygen solution rates in a culture and the sulphite solution enables one to predict from the sulphite measurements the aeration conditions required to satisfy the oxygen demand of a culture.

The rate of oxygen transfer from gas to liquid, that is the oxygen solution rate, in an aeration system may be determined by measuring the rate at which sodium sulphite solution containing copper ions is oxidized in the vessel (Cooper, Fernstrom & Miller, 1944). This method is widely used to measure the aeration efficiency of culture vessels. But the oxygen solution rate in sodium sulphite solution is not necessarily equal to the maximum possible rate in a different absorbing fluid. Although the rate in a sodium sulphite solution is independent of the concentrations of sulphite and catalyst over wide ranges, changing the catalyst from copper to cobalt greatly alters it (Phillips & Johnson, 1956; Pirt, Callow & Gillett, 1957). The physical theory of gas absorption with chemical reaction is complex and still being developed (Sherwood & Pigford, 1952), and recently Schultz & Gaden (1956) have considered the more fundamental aspects of oxygen absorption by sodium sulphite solution. This paper is concerned with the empirical relationship between the maximum oxygen solution rate in a bacterial culture and the oxygen solution rate given by the sulphite method.

Attempts to correlate oxygen solution rates in sodium sulphite solution with those in cultures have been made by Maxon & Johnson (1953) and by Pirt (1957). Maxon & Johnson (1953) compared a yeast culture with the sulphite system and found that the ratio of the maximum oxygen solution rates in the two systems under the same aeration conditions was close to unity. Pirt (1957) working with *Aerobacter* found that the ratio lay between 0·5 and 1·0. In both cases the oxygen solution rates were determined indirectly and involved some assumptions. We have now determined directly the maximum possible oxygen solution rates in a culture of bacteria and compared these with the rates obtained in sodium sulphite solutions. Knowing the quantitative relationship between the oxygen solution rates in the two systems under

the same aeration conditions, one can predict from sulphite oxidation rate measurements the aeration conditions needed to satisfy any oxygen demand of the culture.

Methods

Oxygen solution rate in sodium sulphite solution

The rate of oxygen uptake of sodium sulphite solution containing copper ions (σ_s) was measured by the method of Cooper *et al.* (1944) with the precautions given by Pirt *et al.* (1957).

With increase in temperature from 25° to 40°, σ_s increased linearly by 13%. The σ_s values were, where necessary, corrected for temperature changes.

Maximum oxygen solution rates in cultures

Rates of oxygen uptake in continuous cultures of *A. aerogenes* (σ_c) were measured by the method of Pirt & Callow (1958*a*). The measurements were made during the study of 2:3-butanediol production by the bacteria. To determine the maximum possible oxygen solution rate, σ_c must be limited by the oxygen solution rate and not by the ability of the culture to take up oxygen; results which show that this was so are given in Fig. 1. The σ_c value, therefore, was the maximum possible oxygen solution rate in the culture for the particular aeration conditions.

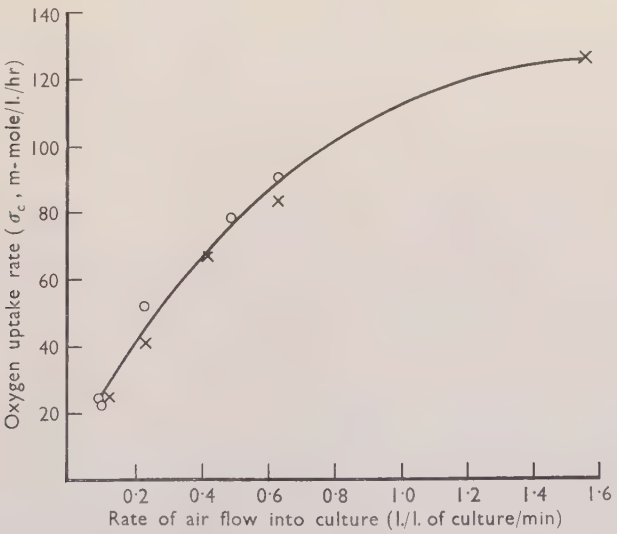

Fig. 1. The relationship between rate of oxygen uptake and rate of air flow into the culture. The rate of oxygen uptake was clearly limited by the rate of air flow into the culture. Open circles, dilution rate 0·1 hr^{-1}; crosses, dilution rate 0·2 hr^{-1}. An impeller of diameter 63 mm and blade height (below disc) 9 mm, was used at 1000 rev/min.

The term 'same aeration conditions' means the same values for the stirrer speed, the rate of air flow *into* the vessel, the gas pressure in the vessel and the volume of

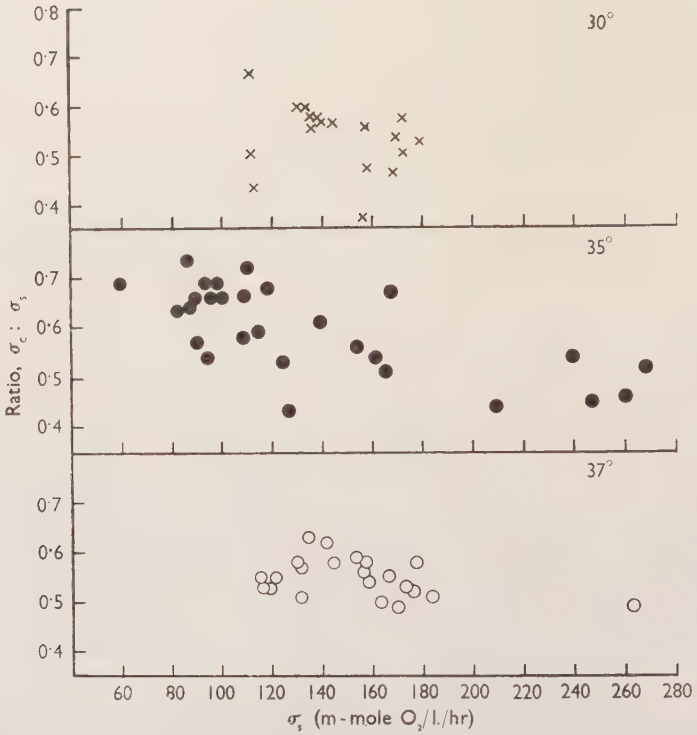

Fig. 2. The ratio of oxygen solution rate in the culture (σ_C) to the oxygen solution rate given by the sulphite method (σ_S), plotted as a function of σ_S.

liquid in the vessel. The gas pressure in the vessel was slightly (less than 75 mm water gauge) above atmospheric pressure.

The antifoam agents used in the cultures were: Alkaterge-C (Commercial Solvents, Terre Haute, U.S.A.), 30% v/v in liquid paraffin, and a silicone emulsion, Antifoam 60 (General Electric Company, New York). It was the practice to add the antifoam after a foam layer had formed on the surface of the culture (Pirt & Callow, 1958*b*).

Results and Discussion

The ratio of oxygen solution rate in a culture to that found by the sulphite method

Graphs of the ratio σ_c:σ_s at different σ_s values are shown in Fig. 2. Its value depended on σ_s and ranged from 0·7 at low values of σ_s to 0·5 at high values. The significance of the variation was estimated statistically. The correlation coefficients between the value of the ratio and σ_s were calculated for the data obtained at 30°, 35° and 37°. The 35° data had a highly significant negative correlation coefficient; the 37° data had a fairly significant (5% probability) negative correlation coefficient; the 30° data had a negative coefficient but it was not significant. Thus, on the whole, there was a significant drop in the ratio with increase in σ_s.

The independence of the ratio σ_c:σ_s of bacterial concentration and some other factors

Since the bacterial concentration usually increased with increase in σ_s the fall in the ratio with increase in σ_s could have been due to increase in the bacterial concentration. One can eliminate the effect of changing bacterial concentration by taking the partial correlation coefficient. The partial correlation coefficients between the ratio and σ_s, eliminating the effect of bacterial concentration, were significantly negative in the cases of the 35° and 37° data and negative but not significant in the case of the 30° data. This indicates that the fall in the ratio with increase in σ_s was not accounted for by change in the bacterial concentration. In support of this is the fact that the partial correlation coefficients between bacterial concentration and the ratio, eliminating the effect of varying σ_s, were not significant.

The evolution of CO_2 by the culture might be expected, by diluting the oxygen, to decrease the oxygen solution rate. In our cultures the dilution of oxygen by CO_2 was appreciable, since CO_2 partial pressures up to 0·25 atmos. (Pirt & Callow, 1958*a*) were reached in the effluent gas. However, the partial correlation coefficient of CO_2 pressure and the ratio, eliminating the effect of varying σ_s, was not significant; therefore it was concluded that the CO_2 evolution did not affect the oxygen solution rate in the culture. Variations in temperature in the range 30–37°, in pH value in the range 4·5–7·0, and in specific growth rate in the range 0·1–0·2 hr^{-1} also had no appreciable effect on the ratio.

The effect of antifoam agent

There are somewhat conflicting reports about the effect of antifoam agents on oxygen solution rates. Deindoerfer & Gaden (1955) and Corman *et al.* (1957) have reported that antifoam agents reduce oxygen solution rates. However, the effect noted by Corman *et al.* (1957) may have been due to chemical inhibition of the sulphite oxidation. In contrast, Chain & Gualandi (1954) found that the occurrence of foaming in a culture medium decreased the oxygen solution rate and that the addition of antifoam then increased the oxygen solution rate.

We did not determine oxygen solution rates in cultures in the absence of antifoam because it was essential to add antifoam to prevent foaming. We found that the ratio σ_c:σ_s was the same whether the silicone or the Alkaterge antifoam agent was used. The spread in the values of the ratio under any particular aeration conditions may have been due to variation either in the amount of antifoam present or in the degree of suppression of foaming.

The meaning of the dependence of the ratio σ_c:σ_s on σ_s

It has been shown above that the value of the ratio σ_c:σ_s ranged from 0·7 at low values of σ_s to 0·5 at high values. This means that to satisfy the oxygen demand of the culture the aeration conditions must be such that the oxygen solution rate determined by the sulphite method is 1·4 to 2·0 times the oxygen demand. A method of calculating the oxygen demand of a growing culture has been given by Pirt (1957).

We wish to express our thanks to Mr. S. Peto for his advice and assistance in the statistical analysis of the results. Mr. J. E. D. Stratton has rendered invaluable technical assistance.

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OBSERVATIONS ON FOAMING AND ITS INHIBITION IN A BACTERIAL CULTURE

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SUMMARY: Inhibition of foaming in a continuous culture of bacteria has been studied. At first, foaming was inhibited by the addition of antifoam to the culture only when a foam layer was present. When the antifoam was added in this way foaming often became intense and antifoam additions had to be more and more frequent. In a preferred method the antifoam was added at regular intervals which were sufficiently short to inhibit foaming completely throughout the intervening periods. This method required less antifoam than the other. The effects of foaming in cultures, its causes, and allied problems are discussed.

The development of much more efficient agitation and aeration systems for fermentation processes has made foam control a serious problem. Unless foaming is suppressed it can have very adverse effects (Ghosh & Pirt, 1954), and in our study of the 2:3-butanediol fermentation (Pirt & Callow, 1958) control of foaming was a major problem. We used conventional control methods; that is, addition of antifoam agent after a foam layer had formed and also mechanical foam breaking vanes attached to the stirrer shaft. The latter method was of little value, but if a large quantity of foam does form it can assist foam breakdown by a chemical antifoam.

Reviews and contributions to knowledge of foam inhibition have recently been made by Ross (1950), Bikerman (1953) and Gaden & Kevorkian (1956).

It was difficult to improve the method of foam control because antifoam action has little theoretical basis, most of the large number of antifoam agents available have not been evaluated quantitatively or qualitatively under diverse working conditions, and little is known about the causes of foaming in fermentations. By a different approach to the use of chemical antifoam agents we effected a dramatic improvement in foam control in continuous fermentations.

Methods

Antifoam preparations. These were: Alkaterge-C (Commercial Solvents, Terre Haute, Indiana, U.S.A.), 30% v/v in liquid paraffin; silicone Antifoam A, 70% v/v in silicone fluid MS.200/10 C.S., both supplied by Midland Silicones, 19, Upper Brook St., London, W.1; Antifoam 60, a silicone emulsion supplied by the General Electric Company, New York, which was diluted to 30% v/v with water. They were sterilized by autoclaving for 20 min at 126°. Some batches of Antifoam 60 and other makes of silicone emulsion we tried were not stable to autoclaving. The silicone emulsions were also subject to microbial decomposition during storage. Some of the silicone

emulsions marketed, it should be noted, are not suitable for fermentation work because they contain chemicals added to inhibit the growth of micro-organisms.

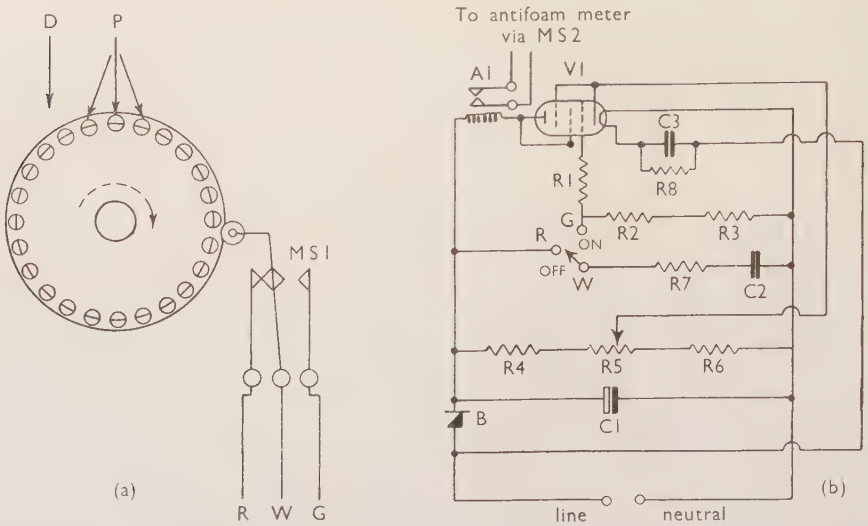

Fig. 1. A device for automatic operation of the antifoam metering pump described by Elsworth *et al.* (1956).
(a) This is a time switch to operate the metering pump at fixed intervals. The 4 in. diam. circular Tufnol disc D, driven by a clock motor (1 rev/12 hr), carries 24 equally spaced brass posts (P), $\frac{1}{4}$ in. diam. and $\frac{1}{2}$ in. high, with screwdriver slot in head and partly cut away on one side. The post may be rotated in its hole so that it depresses (cut away portion facing inwards) or fails to depress (cut away portion outwards) the microswitch MS1. Thus the contacts W and G of the microswitch may be closed from a minimum of one to a maximum of twenty-four times in 12 hr, the time lapse between two posts being $\frac{1}{2}$ hr. In the present device MS1 and MS2 (Fig. 1b) replace the manually operated switch Q and the associated switch R in Fig. 9, p. 276, of Elsworth *et al.* (1956).
(b) This electronic circuit is used to prevent repeated operation of the metering device during the several minutes that MS1 is depressed by a post on the slowly moving disc.
The relay A1 operating MS2 becomes energised when the contacts W and G of MS1 close and allow the charged capacitor C2 to discharge through the valve. The relay remains closed for about 5 sec, depending on the values of R1, R2 and R3, and operates MS2 during this period. After this, no further action takes place until the moving post is clear of MS1. Then contacts R and W close and C2 is recharged ready for the next cycle of operations.
Components. Resistors: R1, 220 KΩ, $\frac{1}{2}$ W; R2, 100 KΩ, $\frac{1}{2}$ W; R3, 2·2 MΩ, $\frac{1}{2}$ W; R4, 100 KΩ, $\frac{1}{2}$ W; R5, 10 KΩ, $\frac{1}{2}$ W, variable; R6, 4·7 KΩ, $\frac{1}{2}$ W; R7, 10 KΩ, 2 W; R8, 1 MΩ, $\frac{1}{2}$ W.
Condensers: C1, 8 μF Electrolytic, 350 V working; C2, 2 μF Paper; C3, 4 μF Paper.
B, Metal rectifier, DRM 2B, 250 V, 100 mA. V1, valve E.F.50.
A1, G.P.O. relay Type 3000, 2 KΩ.
MS1, Burgess roller type microswitch, single pole centre off.
MS2, Burgess roller type microswitch, single pole on/off, normally on.
R, W and G, contacts of MS1.

Methods of adding antifoam. Continuous cultures of *Aerobacter aerogenes* were grown in the apparatus of Elsworth, Meakin, Pirt & Capell (1956). The stirrer speed was 1000 rev/min. The different intensities of aeration are expressed in terms of the

oxygen solution rate (σ_s, m-mole/l./hr) determined by the sulphite method with copper ions as catalyst. The corresponding air flow rates may be found by reference to the paper of Elsworth *et al.* (1956). The volume of the culture was 2 l. The dilution rate was 0·1–0·2 hr^{-1}.

Antifoam was added to the culture through the motor-operated metering valve. A single addition of antifoam consisted of 2–4 drops, that is, *c.* 0·15 ml. In 'manual' addition of antifoam, the valve motor was switched on by hand and one addition of antifoam was made whenever a layer of foam about 2 cm thick was present on the surface of the culture. Manual addition of antifoam, of course, required constant supervision of the apparatus. Periodic addition of antifoam was effected by means of a valve automatically operated at fixed intervals by means of a time switch, which may be used to operate more than one antifoam valve. The circuit is shown in Fig. 1.

Results

The observations reported here were made during the course of about 15,000 hr operation of continuous culture apparatus in runs ranging from 200–2000 hr. The fermentation process was 2:3-butanediol production by *A. aerogenes*. Other aspects of the fermentation have been reported elsewhere (Pirt & Callow, 1958).

Quality of antifoam: 'normal' and 'intense' foaming

Alkaterge-C was the most effective antifoam in that the required amount was several times less than the necessary amounts of the other antifoams.

With 'normal' foaming one addition of Alkaterge-C at 4–12 hr intervals was sufficient to control foaming with σ_s values up to 170. Thus the average concentration of Alkaterge-C was *c.* 200 p/m (v/v). Occasionally, with manual addition of antifoam, foaming spontaneously and rapidly became intense. When this 'intense' foaming occurred the frequency of antifoam addition was about ten times that with normal feeding, so that on these occasions the concentration of Alkaterge-C reached about 2000 p/m (v/v). Intermediate states of foaming between the normal and intense were rarely observed.

The effects of foam formation

For 100–250 hr after a culture was started, with σ_s values between 130 and 170, foaming was normal. After this a period of intense foaming always occurred when the σ_s value was 130 or more. Flocculation of the bacteria was associated with intense foaming. It also occurred on rare occasions with normal foaming, but on these occasions it was much less pronounced. The flocculation associated with intense foaming disappeared rapidly when foam formation was reduced to the normal level by lowering the σ_s value.

When intense foaming occurred the rates of carbon substrate utilization and of butanediol production by the culture decreased. The flocculation of the cells could account for this since it would reduce diffusion of nutrients to and products from the cells.

Causes of foaming

Intense foaming usually happened spontaneously and was not correlated with any change in the culture conditions. However, it did seem that foaming was more favoured at pH 7 than at pH 5.

The culture medium (sucrose–mineral salts) did not foam when aerated by itself. Foaming only occurred after the bacteria were produced in the medium. Thus the bacteria may have been the source of the foam stabilizing agent. Another possible cause was considered, namely, that there was some critical concentration of the antifoam agent above which it stabilized the foam instead of breaking it. Ross (1950, p. 10) has mentioned this possibility. Attempts to demonstrate this failed. Bacterial suspensions from cultures which foamed normally were stirred and aerated or mechanically shaken with different concentrations, from 0·02%–10% (v/v), of the Alkaterge-C and paraffin preparation, but foaming was always completely suppressed. In such experiments, also, flocculation of the bacteria never occurred, hence flocculation was not caused by Alkaterge-C (see note added in proof, p. 216).

Prevention of intense foaming

Even with intense foaming addition of antifoam agent always destroyed the foam, but the effect was very short-lived and it became necessary to find better means of preventing intense foaming.

Supposing that this was due to the accumulation of some foam stabilizing substance in the vessel, we decided to see whether washing out the culture vessel's contents would stop intense foaming. The vessel's contents were washed out with water and replaced with fresh medium; this medium was inoculated with a shake-flask culture which had been inoculated with 1 ml of the discarded culture. Thus the carry over of old culture was only 1 ml. But sometimes after this treatment, immediately the bacterial population reached the steady state level (0·5–1% dry wt), intense foaming began again, another fact which suggests that the bacteria were the source of the foam stabilizing agent.

A reliable method of stopping intense foaming was to decrease the σ_s value, to 100 or less. Then the required frequency of antifoam addition rapidly decreased. After a few days under these conditions (the minimum time was not determined) it was found possible to increase the oxygen solution rate again without causing intense foaming.

Obviously the above methods of foam prevention were inadequate. The automatic addition of antifoam agent when foam rises to a certain point (e.g. Pfeifer & Heger, 1957) does not seem to differ in principle from our method of manual addition. In both methods the aim is to *destroy* the foam after it has formed. In our method of periodic addition of antifoam the object was to maintain a sufficient concentration of antifoam in the culture to *prevent* foam formation at all times rather than to destroy the foam after it had formed. It was found that if single additions of antifoam were made at 2 hr intervals to a culture with σ_s values of 130–170 and dilution rate 0·2 hr^{-1}, intense foaming never occurred. Whereas with manual addition of antifoam it was impossible to run for more than 250 hr without a period of intense

foaming, the periodic addition system has been operated for 5000 hr without a single such period. We have also operated the periodic addition system for long periods with 4-hr intervals between additions; in this case there was a slight accumulation of foam, and occasionally an extra antifoam addition was made manually. The total consumption of antifoam in the method of periodic addition has been less than it was in the manual addition method, and it could probably be decreased if the addition were made truly continuous. This would require the development of an extremely slow pumping device.

Discussion

The results show that it is far easier to *prevent* foaming than to destroy the foam after it has formed. The periodic addition of antifoam prevented foaming at all times, and has the advantages that it eliminates the need for continuous supervision of the apparatus; it enables the culture vessel to be filled almost to capacity since there is no need to leave a space for foam accumulation; it prevents adverse effects such as decrease in oxygen solution rate (Chain & Gualandi, 1954), denaturation of protein (Richmond, 1958) and flocculation of bacteria caused by foaming. The association of flocculation of bacteria with foaming seems to be a new finding.

Foaming in fermentations is vaguely attributed to complex substances either present in the culture medium or produced by the micro-organisms. To what extent any particular constituent of the culture such as a protein, a peptide, a fatty acid salt or the microbial cell itself is responsible for foaming seems in no case to have been determined. In the butanediol fermentation it appears likely that the foam-stabilizing agent was either the bacterial cells or a product of them. Isolation and characterization of the foamstabilizing agent would obviously enhance knowledge of the causes of foaming in fermentations. It is conceivable that intense foaming was caused by the selection of a mutant type of bacteria able to metabolize and thus destroy or neutralize the antifoam agent. However, this seems unlikely in view of the fact that intense foaming did not occur when the periodic method of antifoam addition was used.

More quantitative information about the efficacies of antifoam agents and how they are influenced by culture conditions would be of considerable value in the submerged culture of micro-organisms.

Mr. A. L. Dean designed the electronic circuit (Fig. 1b) used in the antifoam meter. The time switch (Fig. 1a) was based on a similar apparatus designed by Dr. D. Herbert. Technical assistance given by Mr. J. E. D. Stratton is gratefully acknowledged.

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(*Received* 1 *May*, 1958)

Note added in proof. An important point to consider in further studies of this subject is that the reaction of non-growing, or resting bacteria to surface-active agents such as Alkaterge-C may be different from that of growing bacteria since entry into the resting phase may result in modification of the bacterial surface.

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THE ADVANTAGES OF A PLATINUM TUBE DROPPING PIPETTE FOR MAKING COLONY COUNTS

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SUMMARY: For routine purposes where a large number of tests is involved the dropping pipette technique has many advantages over other methods of determining the colony count. However, glass pipettes are fragile and much time and labour have to be used in making and calibrating replacements. A robust platinum tube dropping pipette has been found very satisfactory. No difficulty was experienced in obtaining complete sterility of the pipettes in the technique described, and reproducibility of drop size was good.

All methods of making colony counts involve adding a known volume of a suspension to a medium, incubating and then counting the colonies. The advantages of a dropping pipette for this purpose have been recognized for some 60 years. A summary of early work and references is given elsewhere (Davis & Bell, 1959). Of more recent papers mention may be made of those by Pomales-Lebrón & Fernández (1952) and Mallam & Broitman (1956).

Pomales-Lebrón & Fernández (1952) described a method in which volumes of from 0·01 to 0·1 ml were added to 12 ml of medium in a plate, using a 0·2 ml pipette. They considered the method to be economical in time and materials, less liable to errors in counting and through contamination, to avoid the possible harmful effect of hot agar on certain bacteria, and to minimize spreading growth. Mallman & Broitman (1956) used a 1:20,000 buffered saline solution of Triton X 100 (a non-ionic wetting agent) as the diluent. This gave a drop area nearly 30% greater than without the wetting agent and a more even distribution of colonies. In our work the use of the dropping pipette method reduced the number of plates and the incubator space to a quarter of what it would have been with the volumetric pipette method. There was not only a great saving in initial expenditure but also in washing up and in cleaning and sterilizing the plates and test tubes.

The development of a durable pipette

Confronted with an investigation which required large numbers of colony count determinations at frequent intervals, we first considered using the classical Pasteur pipette with the tip calibrated in a no. 54 gauge hole. With all drop techniques each pipette can be used only once; it then has to be cleaned and re-sterilized, and the fragility of the tip of the glass pipette was clearly its biggest disadvantage. Wastage being high, considerable time was needed for making and calibrating replacements.

In an effort to solve the breakage problem Mr. A. Rowlands submitted a pipette formed by grinding a glass stem into the top of a hypodermic needle which had been cut off square. Although the needle functioned well it tended to fall off the glass stem, and the latter sometimes cracked during sterilization. This instrument was, therefore, abandoned, but if these faults could be overcome this type of pipette should be satisfactory. Later, Mr. W. H. Pierce kindly sent us a pipette consisting of a short length of platinum tubing sealed into a bulb pipette. Trials showed it to be a most useful tool and we adopted it for our work. In view of the cost of platinum we examined the possibility of using a substitute metal. Two firms sent us samples of the correct size of tubing in certain metals, and Mr. A. Rowlands prepared pipettes using these materials, but although these pipettes worked satisfactorily they cracked after a short period of use. Further, the cost of the platinum tubing was small relative to that of making the pipette, so that there was no great point in seeking a cheaper metal. Moreover, if the pipette cracks the piece of platinum tubing can be sealed into another pipette.

Description of the pipette

The platinum tube pipette (Fig. 1) consists of a 9 mm length of platinum tubing of 0·9 mm o.d. sealed into a bulb pipette so that 6·5 mm protrude. The tube must be cut off square very precisely, and the top must be flush with the inside surface of the bulb; these points should be emphasized to manufacturers. The angle of the tube is a matter of personal preference; some workers prefer 45° but we prefer the tube to be slightly closer to the line of the pipette, and in the illustration the angle is 30°. The pipette is operated by means of a teat.

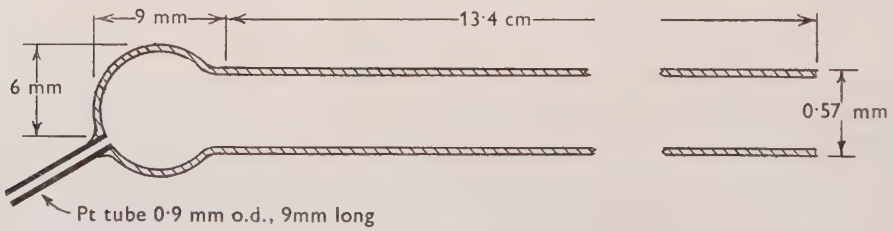

Fig. 1. Details of the construction of the platinum tube dropping pipette.

As with normal Pasteur pipettes, the volume of the drop varies directly with the outside diameter of the tube. The pipettes must be calibrated with the liquid to be used, e.g. quarter-strength Ringer's solution, and the volume (V) of the drop is given by the formula $V=2\sigma r/dg\phi$, where r is the radius of the tip, σ is the surface tension of the liquid, d is the density of the liquid, g is the acceleration due to gravity and ϕ is a correction factor for the neck of the drop.

Special calibration must always be made if the solution contains a detergent or other substance affecting surface tension, as the drop size varies directly with the surface tension of the liquid. Calibration may be effected by allowing a known number

of drops, say twenty, of liquid to fall very slowly into a flask or beaker of known weight and immediately reweighing. We have found the drops to have an average volume of 0·018 ml. With careful use of a single pipette the error is 0·001 ml or about ±5%. Pipettes may vary slightly and if an accurancy of ±5% is required they should be calibrated individually and if necessary adjusted; or they may be put in groups with slightly different drop volumes and therefore having slightly different factors for converting mean colony numbers/drop to counts/ml. It is useful to prepare a table giving counts/ml corresponding to drop counts, as it makes calculation unnecessary.

Method of use

All pipettes are fitted with rubber teats. At least 6 pipettes should be cleaned and stood in a row in a beaker containing 70% alcohol or 75% methylated spirit. The pipette on the left is taken, the alcohol squeezed out and the pipette flamed. About 5 sec is required for the alcohol to burn off and then, after a pause of 2–3 sec, the pipette is used starting with the highest dilution. When all dilutions have been 'plated' the pipette is rinsed in water if necessary and then returned to the right of the row of pipettes in the beaker. Each pipette should be immersed for at least 15 min before re-use. This helps to prevent cracking as well as ensuring a thorough rinsing and sterilization. If the pipette is heated successively at intervals of only 3 min it will tend to crack, but if flaming is done only at intervals of 15 min or more, breakages are rare.

After use the pipette should be rinsed out in hot water and left soaking in 0·5% Teepol overnight. They are then rinsed thoroughly and left in tap water for a few minutes before being placed in a beaker of 70% alcohol ready for use. Sterilization in a hot air oven is unnecessary.

We were concerned with a large scale investigation where the primary interest was the logarithmic increase in numbers of bacteria, and the work was essentially of a comparative nature. For such purposes the drop technique is admirable, and in the interests of speed only single drops were 'plated' from each dilution. Where higher accuracy is required multiple drops should be used.

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THE LOSS OF VIABILITY OF BACTERIA IN SUSPENSION DUE TO CHANGING THE IONIC ENVIRONMENT

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SUMMARY: Following the accidental discovery and subsequent confirmation of an 80% loss of viability in a suspension of *Escherichia coli* when the ionic environment was changed from phosphate buffer to Ringer's solution, and of similar losses after changes in the reverse direction, the behaviour of other organisms was studied. *Clostridium butyricum*, *Streptococcus lactis*, *Escherichia coli*, and *Micrococcus candidus* gave positive results, *Mycobacterium smegmatis* and a brewer's yeast did not. The survey included variations in the conditions, mostly with *E. coli*. Minor constituents of the solutions or alteration of their pH modified the effect, e.g. that of 1% NaCl in place of Ringer's solution or of pH 8·0 instead of the usual pH 7·2, was quite different. Also, the addition of glucose, sodium thioglycollate, or methylene blue diminished it, with one exception in the case of glucose. *Mic. candidus* responded if the solutions were chilled, but not at room temperature. With electrolyte concentrations below about 0·02 M there was no effect. Various other changes of anions were effective, as also was change from a univalent to a bivalent cation. A second treatment, following the necessary intermediate reverse change which had no effect, caused another smaller mortality. This reduced sensitivity gave a diminished loss which persisted through many subsequent subculturings.

The studies here reported followed on the chance discovery of the striking and unforeseen effects on viability of altering the ionic environment of *Escherichia coli*. For an experiment involving a comparison between suspensions in two solutions similarly congenial but containing different anions, the growth on a slope culture of *E. coli* had been washed off in 0·15 M phosphate buffer of pH 7·2 containing 1% of glucose, the suspension divided into two equal parts, and centrifuged; one deposit was then resuspended in Ringer's solution containing 1% of glucose and the other in the phosphate. When the viable cells in the two populations were estimated (in this case by turbidimetry, see below), instead of their values being approximately equal the suspension in Ringer's solution showed a deficiency of 80%. In a repetition, viable cell estimations by plate colony count and by turbidimetry agreed in confirming the loss of viability. Since this finding suggested a possible source of error in bacterial estimations involving different solutions, the phenomenon was investigated further, and the general survey described below illustrates the extent to which it may occur under a variety of conditions, though most of the work has been done with *E. coli*.

Materials and Methods

Organisms, and their cultivation. The organisms used were: *Escherichia coli* 1/49*a* (NCTC), *Clostridium butyricum* (NCTC), *Mycobacterium smegmatis* (NCTC), *Streptococcus lactis*, strain M_2S_1 (Dr. A. A. Nichols) and *Saccharomyces cerevisiae* (as brewer's yeast, Younger & Co., Edinburgh). Two NCTC specimens of *E. coli* were used, the

second obtained $6\frac{1}{2}$ years after the first. The responses of the first had changed little during 5 years but thereafter spontaneously diminished. Specimen 2 gave rise to specimen 3 derived from a single colony. *E. coli* and *Strep. lactis* were maintained as stock cultures, the former in peptone Lab-Lemco broth with transfers at intervals of not more than 14 days, and the latter in litmus milk with transfers at not more than 2-day intervals. All organisms were subcultured daily for a week before an experiment, *Strep. lactis* in yeast dextrose broth, the yeast in Oxoid wort broth (pH 5·2), and the others in the nutrient broth until the last transfer to nutrient agar slopes for test (*Cl. butyricum* in hydrogen). The cultivation temperatures were: *E. coli*, *Cl. butyricum*, and *Mycobact. smegmatis*, 37°; *Strep. lactis* and the yeast, 30°; *Mic. candidus*, 22°.

Suspension fluids

All solutions were made up with distilled water from a 'Manesty' still with a stainless steel condensation tube; they had no other contact with metal.

Ringer's solution. This contained, all as % (w/v) in distilled water: NaCl, 0·9; KCl, 0·042; $CaCl_2$, 0·048; $NaHCO_3$, 0·02; pH, 7·2–7·3.

Phosphate solution. This was double strength (0·15 M) Sørensen buffer, made up of 70% Na_2HPO_4 and 30% KH_2PO_4; pH 7·2–7·3. This had the same electrical conductivity as the Ringer's solution, but its dissociation being about 50%, largely monobasically, its osmotic pressure was little more than half as great.

Citrate solution. This contained 2% (w/v) trisodium citrate, and the pH was 7·5.

Special conditions. The experiments with the yeast were conducted at pH 5·2, the pH of the wort broth, obtained by adjustment with H_2SO_4. When *Cl. butyricum* was used all the solutions contained 0·2% of sodium thioglycollate.

Since in the original experiment the suspension fluids contained glucose (1%), this practice was continued for a time, but its presence was not essential and, as it was a complication, in later work it was usually omitted. Results obtained in the presence of glucose are indicated in the Tables.

Preparation of suspensions and change of environment

Cultures 18 hr old were used, and all manipulations done at room temperature, unless otherwise stated.

To each agar slope culture was added 5 ml of the desired fluid and the growth gently dispersed with the aid of a Pasteur pipette. Broth cultures were centrifuged, and the deposit resuspended in the desired fluid. The washing-off or resuspension took about 10 min. At this stage the suspension contained about 300×10^6 organisms/ml.

Two equal volumes of suspension, usually 20 ml each, were then centrifuged for 20 min at R.C.F. about 2000× gravity and the supernatant at once decanted. One deposit was then resuspended in the original fluid (control organisms), and the other in an alternative fluid (treated organisms), the volumes of fluid being equal and the same as those originally taken. The two deposits were simultaneously dispersed by two operators to preserve uniformity in the times of sojourn in the two fluids, the method being to disperse with a Pasteur pipette into one or more 5 ml volumes of

fluid. Finally, the volume was made up to 100 ml in each case. This resuspension took about 3 min. The suspensions then contained about 50×10^6 organisms/ml.

To minimize the time taken in these procedures the centrifuging was relatively light. This also had the advantage of yielding a loosely packed, easily dispersed deposit, but involved difficulty in decanting the supernatant fluid without loss of organisms. Since the loss could not be assumed to be the same in all cases, the turbidities of the final suspensions were quickly determined, and the necessary correction applied to the viable cell counts. This was done, instead of accurately equalizing the turbidities, in the interests of speed, since it was desired to avoid prolonged sojourn of the organisms in an environment in which the death rate might be notable. The turbidities usually agreed to within 5–10%, but occasionally the discrepancy was greater. In such cases, again for the sake of speed, a rough equalization was made and the counts finally corrected for any remaining discrepancy after a further measurement of the now nearly equal turbidities.

The estimation of viable cells

Two methods were used: the standard plate colony count and a turbidimetric method.

Plate colony count. Serial tenfold dilutions were made and 1 ml quantities plated from several dilutions with 3 or 4 replicates at each dilution. The dilution fluid was usually but not always that used for the resuspension, but in fact this was not important as the reversal of environment occasioned by the use of the first suspension fluid had no effect on viability, as shown below. Dilution and plating of the suspensions of the 'control' and 'treated' organisms was done simultaneously by two operators, and took about 15 min.

With *E. coli*, counts were made on MacConkey's agar after 24 hr at 37°. A comparison count on nutrient agar was once made. Nutrient agar was employed with *Cl. butyricum* (4 days, 37°, under hydrogen), *Mic. candidus* (2 days, 22°) and *Mycobact. smegmatis* (3–4 days, 37°). For *Strep. lactis* the counts were done after 2 days at 30° on yeast dextrose agar, and for the yeast after 3–4 days on malt agar. An example is given in detail in Table 1.

Turbidimetric method. This was used for most of the work with *E. coli* and for some of that with *Strep. lactis*. When parallel samples were examined both by colony counts and by the turbidimetric method (15 cases) the results were in good agreement. However, the latter method has the additional advantage that it provides information about the rate of growth of cells which have survived the change of ionic environment, especially about the immediately post-treatment divisions.

It was realized that if the growth rate of cells which had survived an ion change should prove to be the same as that of cells not subjected to the change, it would be possible to make an estimate of the relative numbers of viable cells in two samples containing cells which had been (a) exposed to the change and (b) not so exposed, the initial numbers of cells being equal. In Table 1 the details are given of a turbidimetric estimation with a parallel colony count estimation of mortality. The graphical work corresponding to the data in Table 1 is given in Fig. 1.

Equal volumes, 5 ml each, of control (no ion change) and treated (ionic environment changed) cell suspensions, or of suitable dilutions thereof, were inoculated into 45 ml of nutrient broth in the case of *E. coli*, or yeast dextrose broth in that of *Strep. lactis*, and incubated at the appropriate temperature. Usually the level of inoculation was of the order of 5×10^6 cells/ml. In the case of the control cells virtually all were assumed to be viable. At suitable standard intervals (usually 90, 120, 150, 180 and 210 min) the growths of these cultures were estimated by measuring the turbidity in a photo-electric turbidimeter of the type described by Müller (1939). This had an effective range of $5–220\times10^6$ *E. coli*/ml and had been calibrated against dilutions of a broth culture whose colony count had been determined on MacConkey's agar. These dilutions were formalinized to stop growth. The growth curves were then plotted, and it became clear that although the treated cell cultures did contain dead cells which distorted the growth curve in the early period, eventually, when the survivors had multiplied to such an extent that the dead cells formed a negligible fraction only of the total population, the growth curves of the control and treated cell cultures became approximately parallel. This is shown in Fig. 1a.

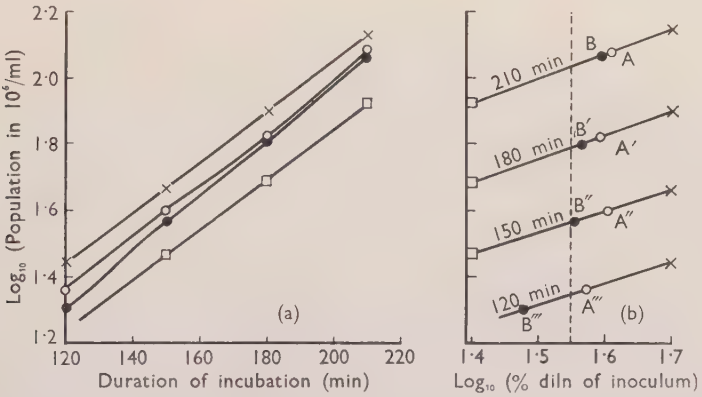

Fig. 1. An illustration of the turbidimetric method of estimating mortality.

(a). The growth curves of cultures inoculated with two dilutions of the untreated cell suspension (no ion change), and with undiluted suspension of treated cells. The growth curve of the treated cell culture, corrected by deduction of the dead cells in the inoculum, is also included. The data are in Table 1.

(b). The interpolation plot corresponding to (a), from which the mortality is determined.
Cultures inoculated with untreated cells: crosses, inoculum diluted to 50%; squares, inoculum diluted to 25%.
Culture inoculated with treated cells: open circles, observed values; full circles, values corrected by deduction of dead cells.
The vertical dotted line lies at the logarithm of the dilution of untreated cells corresponding to the mean percentage survival (36%, 1·55). The survival was also determined on the same suspensions by the colony count method: it was 48% (Table 1).

An estimate of the number of viable cells in the inoculum of treated cells was then obtained by a graphical interpolation method. A diagram was constructed by

plotting, for each standard time when turbidities were measured, the logarithms of the cell populations of the cultures inoculated with control cells against the logarithms of the corresponding dilutions of the inoculum. Through these points a series of lines can be drawn (Fig. 1b). At and after 120 min incubation these cultures will contain only a negligible proportion of nonviable cells, and will, as shown in Fig. 1a (and by special experiments, see below and Fig. 2) be already in the logarithmic phase of growth, so that these lines will be substantially straight and parallel; and ideally, the populations of a control cell culture inoculated with any dilution of the original should, when plotted on Fig. 1b, yield a series of points lying on a vertical line through the logarithm of that dilution. The logarithm of a population of a culture inoculated with treated cells, estimated at a time when it could be assumed that the dead cells introduced with the inoculum represented only a very small, possibly negligible, proportion, was then interpolated on the control culture line for the corresponding time (Fig. 1b, point A), and the equivalent inoculum dilution derived. The treated cell culture could then be regarded as equivalent to a control cell culture inoculated with that particular dilution; this gave directly the percentage of cells which survived the ion change, and hence the percentage mortality.

However, an estimate so obtained has an inherent bias owing to the fact that the inoculum, *c.* 5×10^6/ml, of the treated cell culture did in fact contain some dead cells, so that the equivalent control cell dilution is somewhat underestimated, the more so the higher the mortality and the lower the population of the treated cell culture used for interpolation. A correction taking this fact into account can be made by using the first biassed estimate of mortality to give an estimate of the number of dead cells in the initial population (e.g. an estimated mortality of 60% would imply *c.* 3×10^6/ml dead out of the 5×10^6/ml), and deducting this relatively small number from the population before used (*c.* 120×10^6/ml for point A in Fig. 1b), so as to obtain a corrected value more nearly representing the number of viable cells present (Fig. 1b, point B). From this the corrected values of the equivalent control culture dilution and the percentage mortality follow as before. This process of approximation can be carried further by using this first corrected value of the mortality to obtain a new, slightly larger, deduction, and thence a second corrected mortality, and so on. However, since the deduction can never exceed 5×10^6/ml, such further correction would be significant only if the growth was very small; it has not been needed in any normal case among those here reported.

The treated cell culture populations estimated turbidimetrically at the earlier times can also be used as described above, and can similarly be corrected for the first estimates of dead cells, these corrections being greater the smaller the growth, as shown for the specimen experiment in Fig. 1, a and b. All the corrected values of the percentage mortality (usually four in all) were used to obtain a mean value whose accuracy was determined in the usual way in terms of its standard error. The vertical dotted line in Fig. 1b has been drawn at the position of the logarithm of the dilution corresponding to the mean of the four values of percentage mortality, 1·55. Further, the positions of the interpolated values, which ideally should fall on a single vertical line but in fact do not, give a visual impression of the accuracy of the logarithm of the equivalent control cell dilution, as in Fig. 1b. There is no

significance in the fact that the corrected values in Fig. 1b show a progressive drift in one direction. Sometimes there has been a drift in the opposite direction or, more often, a random scatter on either side of the vertical line. When the mortality is small there is very little departure from the ideal vertical line.

The observations as normally made, with the first after 90 min incubation, give no information about the validity of the assumption that the course of growth of the still viable treated cells would be the same as that of the untreated cells, particularly in respect of the length of the lag phase, which is substantially less than 90 min for *E. coli*. Three special experiments were therefore made with the initial populations increased 20 times, 100×10^6/ml at the start of incubation instead of the normal 5×10^6/ml, so that earlier turbidimetric observations could be made, i.e. after 15, 30 and 60 min; this was at the cost of restricting the incubation to 120 min, after which time the populations exceeded the range of the turbidimeter. The results of one of these experiments are shown in Fig. 2, where the change was from phosphate buffer to Ringer's solution. Clearly, after 30 min the cultures inoculated with untreated cells were already in the logarithmic phase of growth.

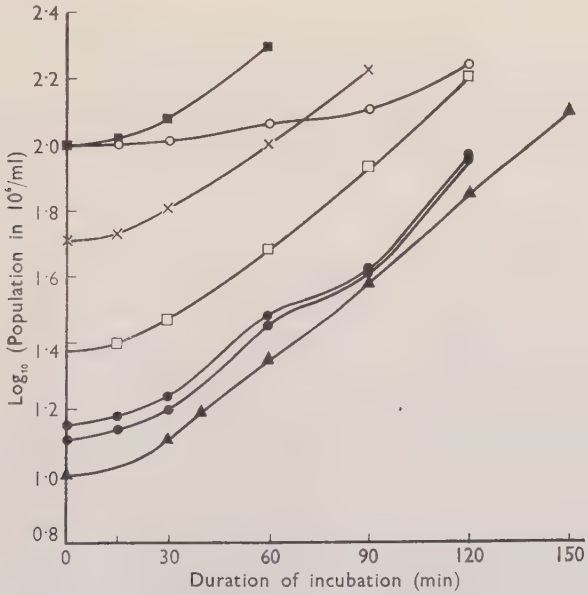

Fig. 2. The growth curves of cultures inoculated with untreated cell suspension and three dilutions thereof, and with undiluted treated cell suspension, together with growth curves for the latter corrected by deduction of estimates of dead cells (see text).
Cultures inoculated with untreated cells: full squares, inoculum undiluted; crosses, diluted to 50%; open squares, diluted to 25%; full triangles, diluted to 10%.
Culture inoculated with undiluted treated cells: open circles, observed values; full circles, upper curve, values corrected by deducting 86×10^6 dead cells/ml; full circles, lower curve, values corrected by deducting 87×10^6 dead cells/ml (see text).
The mortality was also estimated by the colony count method: it was 86%.

The growth curve for the treated cells needed in this case a closer correction than usual for the dead cells present at the start of the incubation, because it was the true shape of the earliest stages of the growth curve that it was desired to reveal. Accordingly, two stages of correction were applied. The culture inoculated with treated cells indicated, from the level of its population at 120 min, an approximate mortality of 75%, i.e. 75×10^6 dead cells/ml were present initially. Making this deduction from the population at 120 min, the first corrected mortality came to 86%. A second correction was then made, in a similar way, and the mortality was found to be 87%. A parallel estimation by the colony count method using the same samples gave a mortality of 86%, in good agreement with the turbidimetric values. Fig. 2 shows two corrected growth curves. The upper was obtained by deducting, not the 75×10^6/ml of dead cells corresponding to the first biassed estimate of mortality of 75%, as would be usual, but the 86×10^6/ml corresponding to the first corrected mortality of 86%. The lower curve was obtained by deducting the 87×10^6/ml of dead cells corresponding to the second corrected mortality of 87%. This further correction was clearly desirable for the earlier stages, though towards the end of incubation its effect was negligible.

The results in Fig. 2, although they required a high accuracy in the observations on the treated cells, were supported by the very closely similar results from the other two special experiments. They show that for *E. coli* there was no marked difference in lag between the growths of the treated cells and those of the untreated controls. This was true for either direction of the change of environment. However, there was a strongly marked wave form in the corrected growth curves of the cultures inoculated with treated cells in the early stages. This virtually disappeared in the later stages, as shown in Fig. 1a.

Results

The lethal effects of change of environment from Ringer's solution to phosphate buffer, or vice versa, on E. coli

An example of the results obtained in a change of the ionic environment of *E. coli* specimen 2 from Ringer's solution to phosphate buffer, in which both the colony count and the turbidimetric methods of estimating survivors were used on the same lots of resuspended cells, is given in detail in Table 1.

The coefficients of variation of the plate colony counts, 8·2% and 11·6%, are representative of those normally obtained, and there has been no tendency for the coefficient to be greater when the environment was changed than when it was not changed. The coefficient of variation of the turbidimetric estimation of survivors, 6%, is less than those of the plate colony counts. This was usually the case; but the coefficients are not really directly comparable.

In this experiment the colony count method indicated a mortality of 52%. This is highly significant, since when the 't' test was applied to estimate the significance of the difference between the means the value obtained was 5·26, giving, for 4 degrees of freedom, $P\ll0{\cdot}01$. This degree of mortality was confirmed by the turbidimetric method (illustrated in Fig. 1, a and b), which indicated 64% mortality.

Table 1. *Details of the determination by plate colony count and by the turbidimetric method of the lethal effect on* E. coli *specimen* 2 *of changing its environment from Ringer's solution (R) to phosphate buffer* 0·15 *M, pH* 7·2 *(P). (See also Fig.* 1)

Plate colony count method	Control, no change	Treated, change
Sequence of environments	R⟶R	R⟶P
Turbidities of second suspensions*	46	44
Plate colony counts (/ml of 10^5 dilution)	249, 187, 221	125†, 83†, 108†
Mean colony count, and S.E.	219±17·9	105±12·2
Coefficient of variation of mean (%)	8·2	11·6
Survival (%), with S.E.		48±7
Coefficient of variation of % survival (%)		14
Mortality (%)		52

Turbidimetric method	Control, no change		Treated, change
Sequence of environments	R⟶R		R⟶P
Turbidities of second suspensions*	46		44
Derived cultures:			
Dilution of suspension used as inoculum (%)	50	25	undiluted†
Populations from turbidity readings after 120, 150, 180, 210 min	28, 46, 81, 136	—, 29, 48, 84	20, 37, 64, 118
Corresponding survivals (%)			30, 36, 37, 39
Mean % survival with S.E.			36±2·2
Coefficient of variation of mean % survival (%)			6·1
Mortality (%)			64

* The same suspensions were employed for both methods.

† Corrected for differences between the turbidities of the resuspensions; the turbidimetric R⟶P populations were further corrected by deducting the approximate number of non-viable cells, 3×10^6/ml, from all four observed populations.

However, it was considered possible, though unlikely, that the effect was not really due to the change in ionic environment as such, but came about because the organisms had markedly different death rates in the two solutions. This was shown not to be so in experiments in which cells were washed off agar slopes as usual with the solution chosen, centrifuged for 20 min, the cells resuspended in the same solution, and samples taken at intervals over a period of 4 hr for estimation of the viable cells by the turbidimetric method, the initial count in each suspension serving as the reference for calculating the mortality.

Table 2. *A comparison of the toxicities of Ringer's solution (R) and phosphate buffer (P) for* E. coli *specimen* 2 *by the turbidimetric method*

Time before sampling (min)	Mortality (%) of cells suspended in			
	R	R+1% glucose	P	P+1% glucose
0	0	0	0	0
15	−1	−1	6	−2
30	9	7	10	4
60	15	18	14	12
120	25	23	25	25
240	35	33	38	34

The results (Table 2) showed that there was no real difference between the solutions tested, though there was a steady death rate in each. While this underlines the necessity for avoiding large differences in time of exposure to different environments, it is clear that small differences do not matter, and in fact the method of working, with two operators, ensured that the differences were small. In addition, it may be pointed out here that the time elapsing between the commencement of resuspension and the inoculation of the culture for viability determination did not exceed 18 min, even with the slower plate colony count method.

It may be remarked here that a general impression of the reliability of the estimations can be derived from the cases reported below in which portions of the same culture were subjected to a graded series of conditions as in Table 2, and some corresponding gradation is to be expected in the results. For the colony count method there are 5 such series each of 3 terms, none of which is numerically out of due order: for the turbidimetric method there are 17 series of between 3 and 11 terms, and these include only two discrepancies.

Using *E. coli*, the environment changes from phosphate buffer (P) to Ringer's solution (R) and vice versa have often been used as standards of comparison for other types of transfer; hence many examples of their effects are available. All of these have been collected in Table 3. The original *E. coli* (specimen 1) was always tested with glucose in the suspension fluids, and consistently during 5 years gave mortalities in the range 75–88% for the P to R change, with a mean and S.D. of $82 \pm 4{\cdot}5\%$. For the reverse change the range was 56–71% (mean and S.D. $63 \pm 5{\cdot}7\%$). The difference between the means is significant (P, $\leqslant 0{\cdot}01$). Another specimen of the same NCTC strain (specimen 2), obtained some $6\frac{1}{2}$ years later, gave mortalities between 45 and 52% with a mean and S.D. of $47 \pm 2{\cdot}6\%$ for the P to R change, and 37–47% for the reverse change (mean and S.D. $42 \pm 3{\cdot}8\%$), both with glucose present. Again, the means differ significantly (P, 0·01–0·02). Without glucose, the mortalities were higher, the corresponding ranges being 57–70% and 46–71%, with means and S.D.s of $64 \pm 4{\cdot}8\%$ and $59 \pm 8{\cdot}5\%$. These means do not differ significantly (P, 0·05–0·10). Thus, the P to R change appeared more lethal than the reverse with both specimens 1 and 2, glucose being present. But this was not borne out by specimen 2 of the same strain when glucose was absent. Specimen 2 was clearly less sensitive than specimen 1 had been to the environment changes tested, though specimen 3, derived directly from specimen 2, behaved just like its parent. During the periods of 5 years for specimen 1 and 1 year for specimen 2 covered by Table 3 there was no evidence of alteration of sensitivity of a particular culture to these environment changes while it was being maintained in the laboratory, but later tests with specimen 1 after an interval of $1\frac{1}{2}$ years showed a change of sensitivity to a value similar to that of specimen 2.

The presence or absence of glucose in the suspension fluids was clearly important; it seemed to exert a protective effect, as in its absence the mortality was significantly higher (P for the differences between means for changes with and without glucose being $<0{\cdot}001$ for both directions of change).

A few experiments were done with *E. coli* specimen 2 to discover whether the age of the culture had any effect, or whether cells harvested from broth behaved

Table 3. *The collected results of experiments with three specimens of the same strain of* E. coli, *involving the environment changes phosphate buffer (P) to Ringer's solution (R) and the reverse*

Specimen of *E. coli*	Mortality (%)§ using the environment changes				Remarks
	P⟶R		R⟶P		
	Glucose, 1%	No glucose	Glucose, 1%	No glucose	
1	79	—	60	—	Results for specimen 1 recorded on the same line were obtained on the same lot of organisms and are thus directly comparable. These cells were from 18 hr slope cultures.
	88†	—	56†	—	
	81	—	58	—	
	—	—	64	—	
	82	—	71	—	
	85	—	—	—	
	83	—	71	—	
	87†	—	66†	—	
	76	—	63	—	
	75	—	57	—	
Mean and S.D.	82±4·6		63±5·7		
2	47 (1)	69	47 (1)	65	With specimen 2, only the pairs of results 'with' glucose, or the pairs 'without' glucose are directly comparable, except for the four results marked ‡ which were all obtained on the same lot of organisms at one time. These cells were from 18 hr slope cultures.
	45 (2)	63†	43 (2)	64†	
	46 (3)	64	42 (3)	—	
	52†	59	37†	—	
	45*	—	37*	46	
	49	61	44	63	
	47‡	70‡	43‡	71‡	
	—	57	—	53	
	—	68	—	64	
	—	65†	—	52†	
Mean and S.D.	47±2·6	64±4·8	42±3·8	59±8·5	
3	—	52 (4)	—	—	With specimen 3 the cells were from 18 hr broth cultures.
	—	57 (5)	—	55 (5)	
2	35	—	30	—	Cells from 7 day slope culture.
	—	56	—	39	Cells from 18 hr broth culture.

§ By turbidimetry, unless otherwise stated. Bracketed pairs of results represent parallel estimations on the same samples. † Results by colony count on MacConkey's agar.

* Results by colony count on nutrient agar for comparison.

(1), (2) and (3): respectively, the 10th, 22nd and 31st transfers from the original freeze dried culture were used. (4) and (5): 1st and 31st days after specimen 3 was picked from a colony of specimen 2.

similarly to those grown on agar. As shown in Table 3, cells from a 7 day agar slope culture seemed more resistant than those from 18 hr agar cultures. Cells from an 18 hr broth culture seemed about as susceptible to the P to R change as those from the 18 hr agar cultures, but were possibly more resistant to the reverse change.

The effects of the P to R and R to P changes on organisms other than E. coli

The results are listed in Table 4. In all cases except one the environment contained glucose (1%) and the mean values for *E. coli* from Table 3 have been included for comparison. *Cl. butyricum* proved as sensitive as the most sensitive *E. coli*. *Strep.*

lactis was not much affected, while *Mic. candidus*, *Mycobact. smegmatis* and *Sacch. cerevisiae* were quite unaffected.

Table 4. *The effect of the phosphate buffer (P) to Ringer's solution (R) environment change, and the reverse, on a variety of organisms*

Organisms	Mortality (%) by the colony count method with the environment* change	
	P⟶R	R⟶P
Cl. butyricum†	84	—
	80	80
E. coli, specimen 1, means	82±4·6	63±5·7
E. coli, specimen 2, means	47±2·6	42±3·8
Strep. lactis, three	22‡	21‡
specimens of	37	37
the same strain	29§	30§
Mic. candidus	0	0
	−9	−2
Mycobact. smegmatis	+4	−1·5
Sacch. cerevisiae	−4	−10

* In all cases, except §, 1% glucose was present.

† With 0·2% sodium thioglycollate present.

‡ Results obtained by turbidimetry.

The effects of variations in the conditions on the lethal effects of changing the ionic environment

Temperature conditions, using R and P. The temperature at which the change of environment was made proved important in the case of *Mic. candidus*, as shown in Table 5, where the P to R and R to P changes, with glucose present, were compared at 3–4° and at room temperature. Exposure at 3–4° produced a marked effect with the P to R change, though not with the reverse change. *E. coli* specimen 1 was slightly, and specimen 2 definitely, more sensitive at the lower temperature, as compared with the mean results obtained at room temperature.

Table 5. *The effect of temperature on the lethal effect of the phosphate buffer (P) to Ringer's solution (R) change of environment and the reverse*

Organism	Method of estimation	Mortality (%), with glucose present, at			
		Room temp., with change		3–4°, with change	
		P⟶R	R⟶P	P⟶R	R⟶P
E. coli specimen 1	Turbidimetry	82±4·6	63±5·7	89	—
E. coli specimen 2	Turbidimetry	47±2·6	42±3·8	63	58
*Mic. candidus**	Colony count	0	0	42	−3
		—	—	40	5
		−9	−2	33	−15†

* Only in the last of the three cases reported were the mortalities at room temperature and at 3–4° obtained for portions of the same lot of organisms.

† This is an extreme case of the tendency of *Mic. candidus* to give a larger count after the change: there were 9 such cases with an average of −5%. This is ascribed to the change helping to disperse the clumping to which *Mic. candidus* is liable.

The effect of pH, using R and P. Phosphate buffer solutions of the same molar concentration but with different pH values were prepared by altering the proportions of the components. The Ringer's solution was altered in pH by adding NaOH or HCl as required, and one set of experiments was done on *E. coli* specimen 2 with Ringer's solution from which the $NaHCO_3$ was omitted.

The results, given in Table 6, show that the lethal effect diminished on either side of pH 7·2, but especially on the alkaline side. Also, when the bicarbonate was omitted from the Ringer's solution the effects were everywhere diminished, the reduction being more marked in the R to P change. In view of these results it is very fortunate that before all the other experiments here reported the pH values of the solutions were always checked and found to be 7·2–7·3.

Table 6. *The effect of pH and the omission of $NaHCO_3$ on the lethal effect of the change from phosphate buffer (P) to Ringer's solution (R), and the reverse, with* E. coli *specimen* 2

Environment	pH	Mortality (% by turbidimetry) with change	
		P⟶R	R⟶P
No glucose. Normal Ringer with $NaHCO_3$	6·0	33	41
	7·2	69	65
	8·0	0	24
No glucose. No $NaHCO_3$ in Ringer	6·0	19	21
	7·2	43	17
	8·0	2	−8

The effect of altering the completeness of the change of environment. In the usual procedure the components of the second solution were in great excess because the small volume of centrifuged deposit from the first suspension was resuspended in a much larger volume of the second solution. To study the effect of less complete changes, centrifuged deposits of *E. coli* were suspended in 10, 20, or 25 ml of the first solution and then mixed with 90, 80 or 75 ml respectively of the second solution, so that after the change of environment the relative proportions of the second and first solutions were as 9:1, 4:1 or 3:1, all very different from the usual proportion. Nevertheless, as shown in Table 7, considerable kills were obtained, the extent of the mortality depending on the degree of change. The retention of phosphate buffer gave more protection against the change to Ringer's solution than did retention of Ringer's solution against the reverse change.

The use of less concentrated suspension fluids. To determine the effect of using environments embodying different concentrations of ions and (inorganic) molecules, experiments were carried out with *E. coli* using various dilutions of the 0·15 M phosphate buffer or of the normal Ringer's solution, as the first suspension fluid, and changing the environment to the corresponding dilution of the other fluid. In the tests with glucose present its concentration was kept at the full 1% throughout each series.

Table 7. *The effect of the extent of the change of environment from phosphate buffer (P) to Ringer's solution (R) and vice versa on the degree of mortality of* E. coli

Specimen of *E. coli*	Proportion of second to first solution after the change	Mortality (% by turbidimetry) with glucose (1%) present in the changes	
		P⟶R	R⟶P
1	Large	82±4·6	63±5·7
	9:1	48	43
	4:1	33	34
2	Large	47±2·6	42±3·5
	9:1	18	19
	3:1	11	23

Table 8. *The effect of concentration of the suspension fluids on the mortality of* E. coli *induced by change of environment from phosphate buffer (P) to Ringer's solution (R) or the reverse*

Specimen of *E. coli*	Concentration of glucose throughout (%)	Concentration of suspension fluids relative to normal	Mortality (%, usually by turbidimetry) in the changes	
			P⟶R	R⟶P
1	1	normal	82±4·6	63±5·7
		1/3rd	50	—
		1/6th (same lot of organisms)	19	21
		1/6th (same lot of organisms)	18*	18*
		1/12th	−5	3
2	1	2×normal	54	50
		normal	47±2·6	42±3·8
		1/4th (all on same lot of organisms)	38	35
		1/6th (all on same lot of organisms)	35	30
		1/9th (all on same lot of organisms)	25	22
		1/12th (all on same lot of organisms)	12	3
2	0	normal	64±4·8	59±8·5
		1/3rd, 1/5th†	5, −1	—
		1/4th, 1/6th†	19, −9	—
		1/8th	−4	—
		1/10th, 1/12th, 1/15th	0, 1, −1	—

* Colony count method. † Pairs of tests on the same lots of organisms.

It appeared, as shown in Table 8, that the lethal effect of the P to R or of the reverse change diminished steadily as the fluids became more dilute, and was virtually abolished for either direction of change at 1/12th the normal concentration, i.e. about 0·01 M, if glucose was present. In the absence of glucose the effect, although initially greater, disappeared even more rapidly and vanished at about 1/5th the normal concentration of the fluids. The maintenance of a relatively high concentration of glucose thus enabled the effect to persist to lower concentrations of the electrolytes.

The effects of O–R poising agents on the lethal effects of the interchange between P and R, and the reverse

Experiments were done with *E. coli*, *Mic. candidus* and *Strep. lactis*, with 0·2% of sodium thioglycollate either in the second suspension fluid, or added to it after the

resuspension had been effected, and other similar experiments were done with methylene blue, adding 2% by volume of a solution of one milk-testing tablet (Oxoid, containing 19 mg of standard methylene blue) in 800 ml of distilled water.

Table 9. *The effects of two O–R poising agents on the lethal effects of the change from phosphate buffer (P) to Ringer's solution (R), and the reverse*

O-R agent	Organism and general conditions	Mortality (% by turbidimetry or colony count) with O-R agent added as stated to the second fluid, and with the changes given							
		Absent		Always present		Added after 15 sec		Added after 10 min	
		P⟶R	R⟶P	P⟶R	R⟶P	P⟶R	R⟶P	P⟶R	R⟶P
Sodium thio-glycollate	*E. coli* spec. 1, with glucose, room temp.	82	71	13	6	23	20	51	53
	Mic. candidus, with glucose, 3–4°	32*	−15*	20*	−5*	26*	−11*	—	—
	Strep. lactis,† with glucose, room temp.	26*	24*	13*	12*	—	—	—	—
Methylene blue	*E. coli* spec. 2. With glucose, room temp.	48	44	16	8	23	21	—	—
	With glucose, 3–4°	63	58	30	7	33	28	—	—
	Without glucose, room temp.	65	63	35	35	—	—	—	—
	Mic. candidus, with glucose, 3–4°	40*	5*	13*	9*	—	—	—	—
	Strep. lactis. With glucose, room temp.	37*	37*	37*	5*	—	—	—	—
	Without glucose, room temp.	29*	30*	17*	3*	—	—	—	—

* Colony count method used. † Solutions were 0·07 M phosphate and 0·5% NaCl.

With both poising agents, when they were present right from the start of the resuspension process the lethal effect of the environment change was generally diminished, the R to P change being most strongly affected. With methylene blue there were, however, two exceptions: with *E. coli* without glucose the R to P change was not more affected than the P to R change, and with *Strep. lactis* with glucose the P to R change was not affected at all. In the few cases where the poising agent was added a little while after completion of the resuspension a diminution of the lethal effect was still obtained, though it was less notable. In the case of *E. coli* specimen 1 a delay of as much as 10 min still left room for a small effect.

In limited trials with *Strep. lactis* and 0·2% sodium thioglycollate in the second fluid the mortality resulting from interchanges between 0·5% NaCl and 0·07 M phosphate buffer (both with 1% glucose), and the reverse diminished the mortalities from 24 and 26%, respectively, to about 13% in both directions of change.

The effects obtained by interchanges other than those between phosphate buffer and Ringer's solution

Since there was no evident reason why interchanges between phosphate buffer and Ringer's solution should have provided the conditions for maximum lethal effects, though, as will be seen, they in fact do so, a number of fluids were tried with various interchanges, the organism chiefly employed being *E. coli*. The fluids included a citrate buffer, pH 7·5, various solutions of inorganic salts, both uni- and bivalent, with and without glucose, and various modifications of Ringer's solution. The univalent metal salt solutions were all assumed to be non-toxic, since the growth of the controls showed the normal relation to the initial turbidimetric measurement of their populations, but the salts of bivalent metals were toxic, and it is therefore convenient to deal with these separately. The Ringer's solution experiments also will be considered by themselves.

Experiments with non-toxic solutions. The results of these are reported in Table 10. With *E. coli* specimen 1 there was no lethal effect with either direction of change between citrate buffer, pH 7·5, and phosphate buffer, pH 7·2. The change from citrate buffer to Ringer's solution was also harmless, but in the reverse change the mortality was 34%. Interchanges in either direction between 1% NaCl and 1% KCl caused no mortality. The substitution of 1% NaCl for Ringer's solution in interchanges with phosphate buffer caused marked differences in the effects. The change from phosphate buffer to 1% NaCl was quite harmless to both specimens 1 and 2 of *E. coli*; thus, if 1% NaCl had been used instead of Ringer's solution in the original experiment no such conspicuous mortality as the 80% then observed would have occurred. The reverse change from 1% NaCl to phosphate buffer did however cause mortalities of 38% and 32% with specimens 1 and 2 respectively. *Strep. lactis* too was sensitive to the change from NaCl to phosphate buffer, in half-strength solutions, but unlike *E. coli* it was also sensitive to the reverse change. Glucose, 1%, was present in all the above trials.

Table 10. *The lethal effects produced by interchanges involving a variety of non-toxic fluids*

Organism	Fluids used*	Code	Interchanges and mortality (% by turbidimetry)†						
E. coli spec. 1	Phosphate buffer+glucose	Pg	Bg	→ Cg	0	Cg	→ Bg	0
	Ringer's solution+glucose	Rg	Pg	→ Bg	0	Bg	→ Pg	38
	1% NaCl+glucose	Bg	Pg	→ Ag	0	Ag	→ Pg	0
	1% KCl+glucose	Cg	Ag	→ Rg	0	Rg	→ Ag	34
	2% Na_3 citrate+glucose	Ag						
E. coli spec. 2	1% NaCl+glucose	Bg	Pg	→ Bg	0	Bg	→ Pg	32
	0·9% NaCl+0·048% KCl	D	Pg	→ Dg	0§	Dg	→ Pg	28§
	D+glucose	Dg	P	→ D	44	D	→ P	38
	D+0·1% glucose	D,0·1g	P	→ Dg	0	Pg	→ Dg	0
	Phosphate buffer	P	P	→ D	28	Pg	→ D	5
	Phosphate buffer+glucose	Pg	P	→ D	29	P,0·1g	→ D,0.1g	12
	Phosphate buffer+0·1% glucose	P,0·1g						
Strep. lactis	0·5% NaCl+glucose	0·5Nag	0·5Nag → 0·07Pg 24‡			0·07Pg → 0·5Nag 26‡		
	0·07 M phosphate buffer+glucose	0·07Pg						

* Glucose concentration 1% except where stated. ‡ Colony count method used.

† Results on the same horizontal line are directly comparable, as are the four bracketed values.

§ These two results were also repeated separately.

The protective effect of glucose is revealed by the results obtained with *E. coli* specimen 2 (see also Table 3 above). The change from phosphate buffer to the mixture of NaCl and KCl (P to D) induced mortalities of 28, 29 and 44% in three trials, but this was eliminated with 1% of glucose in the buffer (Pg), or in the salts mixture (Dg), or in both fluids. In the latter case even 0·1% of glucose reduced the mortality to 12%. However, in the reverse change (D to P) the mortality of 38% in the absence of glucose was reduced only to 28% with glucose in both fluids; the effect of glucose in only one of the fluids was not studied with this direction of change.

Experiments with toxic solutions. In preliminary experiments with solutions of bivalent metal salts there was evidence of appreciable mortality in the transfer from the agar slopes to these solutions. This was confirmed when *E. coli* centrifuged from nutrient broth was suspended to equal densities in 0·85% NaCl, 1% $MgCl_2$, and 1·1% $CaCl_2$, and the two latter suspensions were found by the turbidimetric method to have suffered mortalities, relative to the NaCl suspension, of 10% and 27%. Consequently, in interchange experiments involving such solutions a special 'toxicity control' was carried out. With *both* solutions, organisms were washed from slopes, centrifuged, resuspended in the same (wash-off) solution, and the mortality in the one resuspension relative to that in the other was estimated by the turbidimetric method. This was quite adequate when only one of the interchange solutions was toxic, but when both fluids were toxic the position was very complicated, and only one such trial was carried out, using the same procedure.

The results are shown in Table 11. Taking first the results obtained with glucose always present (experiments 1, 2 and 3), 0·95% $CaCl_2$ was toxic compared with Ringer's solution without the bicarbonate (which was omitted because of an expectation, later shown by turbidimetry of uninoculated solutions to be unfounded, that there would be precipitation of $CaCO_3$), or with the NaCl solutions, the mortalities being about 46, 33 and 37% respectively in the normal period of preparation for an interchange. As expected, the mortality induced by the change from 0·95% $CaCl_2$ to Ringer's solution without bicarbonate, which already contains $CaCl_2$, was negligible. The definite mortality in the reverse change must however have involved a specific interchange effect, since although it was no greater than that recorded in the toxicity control the cells had not been centrifuged in the toxic 0·95% $CaCl_2$, and so had spent much less time in it, in which respect the conditions were in fact comparable with those of the preliminary experiment. In the interchange between 1·0% or 0·85% NaCl to 0·95% $CaCl_2$ there was a notable lethal effect amounting to about 50% in each case. As in the transfer from Ringer without bicarbonate, therefore, this suggests that there was a specific effect due to the change, for in the preliminary experiment the same time of exposure to $CaCl_2$ produced only 27% mortality due to toxicity alone. The reverse change from 0·95% $CaCl_2$ to 1% NaCl induced little mortality but when the NaCl concentration was 0·85% there was 22% mortality, which suggests a specific lethal effect.

The experiments involving 1·0% $MgCl_2$ were all done in the absence of glucose. This solution was less toxic than $CaCl_2$. The change from 1% $MgCl_2$ to 0·85% NaCl led to no mortality. Thus this non-toxic solution had been unable to reverse the toxic action begun by the first exposure to $MgCl_2$. The same is true of the change

from 1·1% $CaCl_2$ to the less toxic 1% $MgCl_2$. In the other direction of change, from 0·85% $CaCl_2$ to 1·0% $MgCl_2$ the mortality was about equal to that in the 'toxicity control'. As argued above for other changes from toxic to non-toxic solutions, this observed mortality indicates some specific effect in addition to the simple toxic action of the second solution. This is also true for the change from the less toxic 1% $MgCl_2$ to the more toxic 1·1% $CaCl_2$; the observed mortalities are too large to be due purely to additive toxic effects.

Table 11. *The lethal effects on* E. coli *specimen 2 of interchanges involving toxic solutions of bivalent metal salts*

No. of expt.	Combinations of fluids	Code	Mortality (% by turbidimetry) due to: Toxicities of fluids		Interchanges			
1*	Ringer without $NaHCO_3$ 0·95% $CaCl_2$	R′g 0·95 Eg	0·95 Eg vs. R′g	46	0·95 Eg → R′g	3	R′g → 0·95 Eg	48
2*†	1·0% NaCl 0·95 $CaCl_2$	Bg 0·95 Eg	0·95 Eg vs. Bg	33	0·95 Eg → Bg	7	Bg → 0·95 Eg	54
3*‡	0·85% NaCl 9·95% $CaCl_2$	0·85 Bg 0·95 Eg	0·95 Eg vs. Bg	37	0·95 Eg → 0·85 Bg	22	0·85 Bg → 0·95 Eg	47
4	0·85% NaCl 1·0% $MgCl_2$	0·85 B F	F vs. 0·85 B	22	F → 0·85 B	0	0·85 B → F	26
5	1·1% $CaCl_2$ 1·0% $MgCl_2$	1·1 E F	1·1 E vs. F	18 36§	1·1 E → F	0‡ 0‡	F → 1·1 E	57 59§

* 1% glucose (g) present in all solutions. † These fluids had equal numbers of Cl^- ions.

‡ These fluids had equal total numbers of ions.

§ Colony count method used, involving a longer time in the toxic solutions than turbidimetry.

Experiments with modifications of Ringer's solution. The results of experiments to reveal the role of bicarbonate, which were carried out on *E. coli* in the presence of 1% glucose, are in Table 12. Evidently the presence of bicarbonate (0·02%) was very important, for its omission from the Ringer's solution completely suppressed the lethal effect of the change to 1% NaCl and the smaller effect of the reverse change, and its presence in phosphate buffer had the same suppressive effect in the changes from Ringer's solution (with or without bicarbonate) or 1% NaCl to phosphate buffer, and the reverse.

Table 12. *The effect of bicarbonate on the lethal effect on* E. coli *of various types of interchange*

E. coli specimen	Fluids used*	Code	Interchanges, and the corresponding mortalities (% by turbidimetry)	
1	1% NaCl	B		
	Ringer's solution	R	R → B 44	B → R 10
	Ringer, no $NaHCO_3$	R′	R′ → B 0	R → R′ 0
	Phosphate buffer	P	P+ → R 0	R → P+ 0
	Phosphate buffer + $NaHCO_3$	P+		
2	1% NaCl	B		
	Ringer, no $NaHCO_3$	R′	P+ → B 0	B → P+ 0
	Phosphate buffer + $NaHCO_3$	P+	P+ → R′ 0	R′ → P+ 0

* There was 1% of glucose present in all the solutions.

The results of experiments on *E. coli* done in the absence of glucose (Table 13) showed that omission of bicarbonate diminished the mortality for interchanges in either direction with phosphate buffer, and omission of the $CaCl_2$ had similar effects in a single trial. Without both $CaCl_2$ and bicarbonate, however, the mortality was not completely suppressed. If anything the double omission was less effective than when only the $CaCl_2$ was absent, but the results are too few and too variable for firm conclusions to be drawn.

Table 13. *The effect of omitting certain components of Ringer's solution (R) on the lethal effect on* E. coli *specimen 2 of the interchanges with phosphate buffer (P)*

Components of Ringer's solution present*				Code	Interchange, and the corresponding mortalities (% by turbidimetry)			
NaCl	KCl	$CaCl_2$	$NaHCO_3$					
+	+	+	+	R	P → R	63±5·7	R → P	59±8·5
+	+	+	0	R′	P → R′	43 43	R′ → P	16 37
+	+	0	+	R″	P → R″	14	R″ → P	22
+	+	0	0	R‴	P → R‴	44 28 29	R‴ → P	38 — —

* There was no glucose present in any of the solutions.

Results on the same horizontal line are directly comparable.

The effect of repetition of a change of environment, with or without growth periods intervening

In this work *E. coli* specimens 2 and 3 were used. Using phosphate buffer (P) and Ringer's solution (R) the effects of repeated P to R changes were studied. Any such repeated interchange must involve three sets of observations, for a change in the reverse direction has to be interposed. To assist interpretation of the results the procedures used should be made clear both for this triple process and for the subsequent study of the descendants of the surviving organisms. For the reverse change enough of the resuspension in Ringer's solution to give a deposit which would at once be resuspended in phosphate buffer was centrifuged. After the reverse change there were two alternatives: in the first experiment (see Table 14) the resuspended organisms were centrifuged from the phosphate buffer, the deposit inoculated into broth and incubated for 4 hr, and this culture used to inoculate agar slopes; the second P to R change was then carried out on the organisms which had grown on the resulting 18 hr slope culture. In the second and third experiments, however, this overnight subculture was omitted, the twice resuspended organisms being centrifuged from the phosphate buffer and at once resuspended in Ringer's solution. *E. coli* specimen 3 was derived from the stock culture of specimen 2 specially for the third experiment. A small colony from a 24 hr MacConkey's agar plate of specimen 2 was picked out, inoculated into broth and incubated for 4 hr, giving a culture from which agar slopes were inoculated. These were then incubated for 18 hr and one of the resulting cultures was used for the first stage of the third experiment.

The survivors of the triple change at the beginning of the third experiment were maintained as a special stock culture; it was regularly subcultured by daily transfers from broth to broth without further treatment. At intervals, samples of this were used to prepare 18 hr slope cultures which were tested in the P to R or R to P changes, resuspensions being carried out in the usual way; this slope culture is included in the number of subcultures listed in Table 14.

Finally, the 38th subculture was used for a repetition of the original experiment (expt. 4), and a broth culture of the survivors was held for 9 days at 37°. This was again maintained with daily transfers, and further tested at intervals up to 19 subcultures.

Table 14. *The influence of reversal and repetition of a change of environment, with or without intervening cultivation on nutrient medium, on the mortality induced by the phosphate buffer (P) and Ringer's solution (R) interchange, together with subsequent tests on samples from a series of subcultures*

E. coli specimen no.	Expt. no.	Culture history	Direction of change, no. of intermediate subcultures‡ and mortality (% by turbidimetry)				
			1st change P⟶R	2nd (reverse) change R⟶P	No. of subc.‡	3rd change (repeat of 1st) P⟶R	Test of subc. using rev. change R⟶P
2	1	Not previously exposed to change	64	−5	1	22	—
2	2	Not previously exposed to change	59	−5	0	11	—
3	3	Derived from a single colony of specimen 2. Not previously exposed to change	52	−20	0	15	
					1	4	—
					3	0	—
					6	−3	—
					10	12, 11*	—
					15	12	—
					18	10	—
					26	12	15
					30	17	22
					38	13	—
3	4	The 38th subculture after first triple change in expt. 3†	13	0	0	−3†	—
					3	10	—
					13	20	—
					19	20	—
2	5§	Not previously exposed to change	R⟶P 46	P⟶R −1	0	R⟶P 9	—

* Estimation by the colony count method on the same lot of organisms.

† The survivors of this triple change (expt. 4) were grown in broth for 9 days at 37° before daily transfers were recommenced.

‡ Including the final agar subculture.

§ The direction of each change was the opposite of that in expts. 1–4.

The results of the four experiments, together with those of the study of the descendants of the survivors from the third, have been collected into Table 14. In the first two experiments the intervening reverse change had no significant effect; with specimen 3 there appeared even to be a 'negative kill' of 20%, indicating that some of the non-viable cells had been revived. After the reverse change the succeeding repeat of the P to R change had a lethal effect which was small compared with that obtained when the P to R change was first applied to the original culture and was

of the same small order whether or not it was preceded by an overnight subculture. In the last experiment (no. 5) shown in Table 14 the directions of the changes, starting with R to P, were the opposite of those in the other four experiments, but the results were very similar.

The tests on the subcultures of the survivors in the third experiment, on the single colony specimen 3, showed the diminished effect persisting during 38 subcultures, being indeed very small during the first 6 subcultures, but later varying irregularly between 10 and 17%. On two occasions an additional test made with the reverse, R to P, direction of change gave similar small effects. After the 38th subculture the repetition of the P to R change was again carried out. The lethal effect of the first P to R change was 13%, that of the reverse change was again zero, but that of the second P to R change was further diminished to a negligible figure. Holding the survivors in broth at 37° for 9 days, as described, and then resuming subculturing and testing showed the lethal effect increasing first to 10% and then to 20% at the 13th and 19th subcultures. Thus the effect of repeating the P to R change of environment was to produce a culture which persisted over a considerable period in showing lethal effects far less than those found after single P to R changes.

Discussion

It is evident that the initial occasion was peculiarly favourable, both as to the solutions used and as to the state of the organisms, for the demonstration of the lethal effect of changing the environment. However, with the use of quarter-strength Ringer's solution, as is more usual for bacterial suspensions, the effect on the viability might well be too small to attract attention. Again, it is more usual, when suspensions in different solutions are required, for separate portions of the same culture to be used for each, and with that technique the possibility of mortality due to change in ionic environment is non-existent. It is in a sequence of operations involving a change of ionic environment that error can arise. Careful attention has been given to all the details of procedure, and although these have been independently scrutinized in action several times no errors of omission or commission have been observed. The possibility that the change of environment introduces some new factor into the process of estimation has been considered, but the experimental evidence seems to be adverse. In particular, differential clumping or light scattering is unlikely to be a major factor wherever changes in opposite directions have both given positive results. Further, it does not seem that the estimations themselves would have been so variously affected by the changes in conditions which have been studied. Tentatively then, it may be assumed that there is a genuine loss of viability with certain changes of ionic environment. This loss, however, may be reversible for a time, for viability can be restored if thioglycollate or methylene blue is added in time.

On the biochemical implications of the loss of viability little comment can here be attempted. The action seems to be in some sense oxidative, since with several organisms sodium thioglycollate counteracted the lethal effect. Glucose also frequently diminished the mortality. That methylene blue in the oxidized form counteracted the effect suggests that possibly the postulated oxidative action concerns cytochrome and that, as in the case of poisoning by cyanide, the autoxidizable

methylene blue may have taken over the lapsed functions of the cytochrome (Baldwin, 1952). Here, too, it is relevant to point out that a purely physical condition in the environment, namely a lowering of the temperature in both solutions, influenced the effect on *Mic. candidus*, which indeed responded only when the solutions were chilled. This also may be connected with the cytochrome respiration (Keilin, 1925).

A first step towards tracing the general course of events may be made from the physical side by considering the ionic environment in terms of the average distance between ions. In 1% NaCl, in the first place, since 1 gramme-molecule contains $6{\cdot}2 \times 10^{23}$ molecules, the spacing between Cl^- and Na^+ ions in 1% NaCl, i.e. 0·17 M, will be 17 Å; in Ringer's solution or 0·15 M phosphate buffer it will be very little different. On the surface of the organism, its negative charge as revealed by electrophoresis corresponds to a calculable surface density of charge and to some arrangement of discrete charges, such as those on adsorbed anions, which for ionic strength 0·17 M (or 0·15 M for phosphate buffer) are spaced at about 17Å, and at 1/8th of that strength, 0·02 M, at 29 Å. These charges attract cations in the surrounding fluid which form a diffuse cloud densest near the surface of the organism. If this is idealized as an equal number of cations all at the same distance and spaced as the negative charges, we can picture an ionic layer of calculable thickness which will be about 8 Å at 0·17 M, and probably more accurately 22 Å at 0·02 M. The lateral spacing varies inversely as the fourth root and the thickness inversely as the square root of the concentration (McQuillen, 1950). In the double layer therefore the spacings are comparable with those in the body of the solution, though somewhat compressed, but the ions have taken up a definitely orderly laminar arrangement. This arrangement is dynamic, with ions coming and going in a random manner, and to this the organism must be adapted. The change of environment involves, by a kind of mass action, substituting for the random variations in the layer an ordered change in which all over the organism ions are replaced by others of a different kind. From the experimental results it is then to be inferred that to such a course of events the organisms may not be adapted, and viability may be lost. When bivalent cations are substituted the surface charge is altered, and the change will clearly be drastic (Beumer, Dirkx & Beumer-Jockmans, 1957). More generally, when we recall that either solution is itself congenial, a possible cause of the breakdown of adaptation is to be sought in the rapidity of the change, and the small and similar ionic spacings in the double layer and in the body of the solution in this particular range of concentrations suggest that this change may be very rapid, even though the environment may not have been completely altered.

However, the existence of a threshold of concentration implies the existence of some factor capable of controlling this rapidity. At this point the water molecules as components of the system have to be considered. At 0·17 M the spacing of 17 Å between ions leaves room for only 4 or 5 water molecules, which in normal water are spaced at 3·44 Å but could be close-packed to 2·8 Å (Bernal & Fowler, 1932); thus all water molecules are within 2 or 3 diameters of an ion. At such distances the strong fields of the ions would modify or prevent the normal quasi-crystalline interlocking of the water molecules while leaving them some translational freedom (Feates & Ives, 1956). Reducing the concentration to 1/8th, i.e. to 0·02 M, would

approximately double the interionic spacings, and leave room for 12 to 15 water molecules, and it should then be possible for the beginning of interlocking to set in, reducing the rapidity of the exchange of ions.

The question thus arises whether it is the suppression of the interlocking above a critical concentration round about 0·02 M that permits electrolytes stronger than this to be effective, not only on the viability in changes of ionic environment, but also in three other recently reported cases in all of which a similar limit is cited. These refer to 'phage action (Jesaitis & Goebel, 1953), to suppressing hydration of D.N.A. (Jacobson, Anderson & Arnold, 1954), and to maintaining enzyme activity (Beers, 1957). Conversely and speculatively, it may be the stronger electrolytes that make general biochemical activities possible, as for instance in the nutrient media here used where the electrolyte concentration is about 0·1 M.

Among the results here presented there is one that calls for special explanation; this is that in the presence of 1% of glucose there is a larger kill of *E. coli* at electrolyte concentrations of about 0·02 M than in its absence, whereas at the usual 0·17 M level it diminishes the kill. Now the spacing of the glucose molecules is 30 Å, so that at 0·02 M there can be one of them between any pair of ions. More than that, they are quite large, about $6\times6\times3$ Å, so that spaced at 30 Å they must themselves have some influence on the water structure. Hence it may be that, if in their absence it is the impeding of the ionic exchange by the incipient restoration of the interlocked water structure that is responsible for the threshold at about 0·03 M, their presence may suppress the interlocking, and lower the threshold to below 0·02 M, as observed. The reversal at low ionic concentrations of the usual glucose effect, which may be chemical, could on these grounds be explained by a countervailing physical action.

Finally, two points of more immediately biological interest will be considered. It can be seen in Fig. 2 that the growth curves for the treated cells, both the observed and, more clearly, the corrected, have the form of a wave. This, since it appears very similarly in the other two special experiments of the same kind, is taken to be a real effect, and may be tentatively ascribed to a transient synchronization of the cell divisions by the shock of the ion change. However, these apparent stimulations and retardations of growth in the broth cultures do not affect the determination of mortality; they must compensate each other without any ultimate loss of viability since the parallel estimations made by the two methods, turbidimetry and colony count, agree well without, on the average, any bias. That this 'wave-effect' may persist to a rather later stage of incubation than is reached in Fig. 2 is suggested by the perceptible wave form of the growth curve of the treated cells shown in Fig. 1a.

The second point relates to Table 14. The observations there reported of mortalities during repeated changes of environment and during the subsequent subcultures of survivors are too scanty to form a basis for firm conclusions; nevertheless, since the series of subcultures, as far as it has been carried, showed a persistently heritable increase in resistance to the lethal effect a tentative interpretation should be attempted.

Two alternative ideas will be considered; the first is that the population of the original suspension was not homogeneous, and that only the more resistant cells

survived the first treatment; the other idea is that the initial change of environment impressed on the surviving cells an altered ability to resist further changes.

The complete elimination of mortality in the intermediate reverse change shows that the cells to which it was applied have different properties from untreated cells on which a first change in this direction would have a marked lethal effect.

Comparing the results of experiments 2 and 3 with those of experiment 1, these results are so similar that a presupposed inhomogeneity cannot be in respect of different phases of the life-cycle, for in experiments 2 and 3 the cells subjected to the final treatment are the actual survivors from the first and second, whereas in experiment 1 they were from a subculture in which all phases of the life-cycle would again be represented.

Comparing now the results of experiment 3, in which the culture had been freshly raised, as described, from a single colony, with the similar results of experiments 1 and 2 using stock cultures of *E. coli* specimen 2 maintained for many months, the implication is that the untreated single colony culture of *E. coli* specimen 3 had rapidly developed closely the same degree of inhomogeneity as had been established in *E. coli* specimen 2. However, considering the series of subcultures of the treated survivors of experiment 3 there is no sign of any comparably rapid development of inhomogeneity though there are fluctuations of mortality in tests using either direction of change of environment. Hence the double treatment has resulted in the loss by *E. coli* specimen 3 of the property of rapidly developing inhomogeneity which it had seemed to possess. Thus the difficulty attending the ready acceptance of the hypothesis of selection from an assumed inherent inhomogeneity is that to that initial assumption we have had to add a second assumption about the speed of development of inhomogeneity in an untreated single-colony culture, and that this latter assumption does not hold for twice treated cultures derived from it.

The alternative hypothesis involves ascribing the persistence of induced resistance, demonstrated as a culture property, to a corresponding induced heritable property of individuals. On this equally tentative alternative the persistence would be supposed to represent in both specimens 2 and 3 an induced change in the *E. coli* similar to that which was described as having occurred spontaneously in *E. coli* specimen 1 after more than 5 years, and showing in the series of subcultures some signs of spontaneous variation, while the repetition in experiment 4 of the triple change of environment after the 38th subculture produced no further change in properties.

On the analogy with recent expressions of general views on heredity in micro-organisms which propagate by binary fission (Hinshelwood, 1956), the change of environment would in this case be supposed to initiate some change starting at the surface of the organism as a result of the changes in the ionic double-layer already discussed, this change, e.g. of molecular structure, being perpetuated as the area of the surface membrane increases before fission and so appearing in both descendants.

However, this alternative hypothesis is suggested only because of the numerical difficulties confronting that of selection; as already stated, more work is needed to discriminate between them.

This work has carried me into unfamiliar fields; I am therefore deeply indebted

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THE POTASSIUM UPTAKE OF *LACTOBACILLUS ACIDOPHILUS*

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SUMMARY: A reproducible flame spectrophotometric method for the determination of the potassium uptake of *Lactobacillus acidophilus* is described. The potassium uptake curve was characteristic and had a coefficient of variation between replicate samples at peak uptake of approximately 2%. Two general applications of the technique have been demonstrated: the evaluation of the toxicity of an inhibitory substance (fluoride as sodium fluoride), and determination of the effect of the interaction of *L. acidophilus* and *L. casei* in a mixed culture.

Since the recognition of the chemoparasitic etiology of dental caries (Miller, 1890), considerable research has been devoted to the morphology, cultural characteristics and physiology of the various members of the oral flora. Probably *Lactobacillus acidophilus* has received the most searching scrutiny because of its statistical correlation with the incidence of dental caries (Davis, Hayward & Bisset, 1956; Hadley, Bunting & Delves, 1930; Collins, Jensen & Becks, 1942). Unfortunately, many of the reports by different investigators cannot be compared because of rapidly occurring metabolic changes in the growing cultures. These alterations have led to reports of pleomorphism (Harrison, 1939; Whyte, 1943; Morishita, 1929), and of changes in cultural characteristics (Warner & Arnold, 1941; Canby & Bernier, 1942) and fermentation reactions (Sullivan, Still & Goldsworthy, 1939; Rosebury, Linton & Buchbinder, 1929). Even such a basic procedure as multiple plate counts of *L. acidophilus* in a single pooled saliva specimen has resulted in considerable variation in count (Permar, Kitchin & Robinson, 1946).

Elucidation of the role of *L. acidophilus* in dental caries requires a method of systematically quantitating metabolic variations occurring at different phases in the growth cycle. Techniques for evaluation must be reproducible and sensitive to slight metabolic changes. This paper describes a technique for the measurement of potassium uptake by bacterial cultures which fulfils these requirements, and demonstrates two general experimental applications, namely the evaluation of the toxicity of an inhibitory substance (fluoride as sodium fluoride), and the determination of the effect of the interaction of *L. acidophilus* (ATCC 4356) and *L. casei* (ATCC 11578) in mixed culture. These organisms were selected because both are found in the oral flora, and both have been associated with dental caries (Davis, Hayward & Bisset, 1956; Tilden & Svec, 1952).

Materials and Methods

Quantities of 9 ml of Rogosa's broth (Rogosa, Mitchell & Wiseman, 1951) modified by the omission of potassium monobasic phosphate (although 5–7 mEq/l. of potassium

were present in other ingredients of the medium), contained in sterile calibrated centrifuge tubes, were inoculated with 1 ml of a 24 hr bacterial culture grown in a similar medium and incubated at 37°. At 4 hr intervals thereafter, 5 tubes were removed from the incubator, centrifuged (2500 rev/min for 15 min), and the supernatant fluid decanted. After two saline washes, 1 ml of concentrated nitric acid was added to the packed cells and digestion accomplished in a hot water bath (95–100° for 30 min). The resulting solutions were treated slightly differently, depending on the flame photometer to be used. For a Baird instrument (Baird Associated, Inc., Cambridge, Mass.), 1 ml of a 2500 p/m LiCl solution was added to serve as the internal standard, after which ion exchange water was used to bring all tubes to a 10 ml volume. A butane gas pressure of 7 lb/in.^{2} was used during analysis. For a Beckman DU Quartz Spectrophotometer with Beckman Photomultiplier attachment (Beckman Instrument Corporation, Fullerton, California), 3 ml of ion exchange water, followed by 2 ml of concentrated ammonium hydroxide, were added to neutralize the acid and prevent damage to the flame housing mirror. Ion exchange water was used to bring all tubes to a 10 ml volume. Instrument and photomultiplier settings were as follows: oxygen, 12 lb/in.^{2}; hydrogen, 3·5 lb/in.^{2}; Mu 766; slit 0·05; zero suppress 1, and sensitivity 1.

Results

The reproducibility of this technique was evaluated as follows: 105 tubes were inoculated with *L. acidophilus*, and the potassium uptake determined in quintuplicate at 4 hr intervals for 96 hr (Table 1). The rate of uptake increased and the variability decreased rapidly, reaching respective maxima and minima between 8 and 16 hr. The coefficient of variation from the 12th to the 28th hour was below 2%. New uptake had ceased by 44 hr.

Table 1. *The results of the flame photometric determination of changes in potassium uptake (mEq/l.) with time by* L. acidophilus

Incubation time (hr)	K uptake in 5 replicates					Mean	S.D.*	C.V.*
0	0·155	0·125	0·125	0·125	0·150	0·136	0·0152	11·18
4	0·625	0·666	0·695	0·730	0·635	0·670	0·0433	6·46
8	1·605	1·505	1·605	1·595	1·580	1·578	0·0421	2·67
12	**1·650**	**1·630**	**1·650**	**1·650**	**1·620**	1·640	0·0141	0·86
16	1·330	1·300	1·300	1·330	1·310	1·314	0·0152	1·16
20	0·940	0·920	0·935	0·940	0·940	0·935	0·0087	0·93
24	0·740	0·760	0·770	0·745	0·745	0·752	0·0126	1·68
29	0·525	0·520	0·540	0·530	0·525	0·528	0·0076	1·44
32	0·500	0·510	0·500	0·485	0·485	0·496	0·0109	2·20
36	0·370	0·260	0·370	0·400	0·360	0·352	0·0536	15·23
40	0·100	0·160	0·235	0·280	0·150	0·185	0·0718	38·81
44	0·065	0·010	0·000	0·055	0·010	0·028	0·0297	106·07
48–96	0·000	0·000	0·000	0·000	0·000	0·000	—	—

* S.D., standard deviation; C.V., coefficient of variation.
Bold figures mark the period of peak uptake.

This time-function method was utilized to determine differences of potassium uptake between 5 series of tubes containing 0, 10, 20, 30, or 40 p/m of fluoride (NaF).

At the period of peak uptake of potassium by *L. acidophilus* there was approximately linear inhibition with increasing concentrations of sodium fluoride (Table 2). This inhibition paralleled the decrease in total growth in the cultures as estimated turbidimetrically using a Klett-Summerson colorimeter (Klett Manufacturing Co., New York). A no. 66 red filter was employed, and distilled water was used as the reference for all readings.

Table 2. *The effect of fluoride* (NaF) *concentration on the potassium uptake* (*mEq/l.*) *and growth of* L. acidophilus

Incubation time (hr)	K uptake and growth at fluoride concentrations (p/m) of:									
	0		10		20		30		40	
	K uptake	Growth*	K uptake	Growth	K uptake	Growth	K uptake	Growth	K uptake	Growth
0	0·08	29	0·09	29	0·08	24	0·10	23	0·10	19
4	0·69	81	0·68	63	0·54	60	0·62	60	0·36	47
8	**1·80**	190	**1·58**	168	**1·38**	164	**1·18**	148	**0·96**	128
12	1·45	195	1·45	193	1·33	180	1·16	167	0·87	141
16	1·22	200	1·20	191	1·06	190	1·01	184	0·82	148
20	0·86	200	0·82	191	0·72	—	0·78	167	0·63	153
24	0·56	203	0·55	191	0·51	180	0·55	173	0·62	148
28	0·23	200	0·35	195	0·26	175	0·27	183	0·55	157

* Growth was measured in terms of turbidity with a Klett-Summerson colorimeter.
Bold figures mark the period of peak potassium uptake.

Because of the mutual association of *L. acidophilus* and *L. casei* in the oral flora, and their possible concomitant influence on caries incidence, it is important to investigate the net metabolic function and stability of mixed cultures. The organisms differ considerably in rate and maximum amount of potassium uptake (Shannon & Harris, 1957), which simplified evaluation of the results obtained with mixed cultures. For this study, 4 series of tubes were inoculated, respectively, with 1 ml of cell suspensions, as follows: (1) *L. acidophilus*, (2) a 2:1 mixture of *L. acidophilus* and *L. casei*, (3) a 1:2 mixture, and (4) *L. casei* alone.

Table 3. *The reciprocal influence on potassium uptake* (*mEq/l.*) *in mixed cultures of* L. acidophilus (*LBA*) *and* L. casei (*LBC*)

Incubation time (hr)	Potassium uptake (mEq/l.) in series			
	1 LBA	2 LBA+LBC 2:1	3 LBA+LBC 1:2	4 LBC
0	0·02	0·04	0·05	0·08
4	0·09	0·12	0·12	0·16
8	0·34	0·40	0·49	0·50
12	**0·47**	0·54	0·68	0·87
16	0·46	**0·56**	**0·72**	**0·93**
20	0·31	0·44	0·66	0·82
24	0·23	0·37	0·50	0·62

Bold figures mark the periods of peak uptake.

L. casei in pure culture had an uptake almost double that of *L. acidophilus* at any given time (Table 3); at the period of maximal uptake (16 hr) the figures were 0·93

mEq/l. and 0·47 mEq/l. respectively. Decreasing the ratio of *L. casei* to *L. acidophilus* in mixed cultures (series 2 and 3) resulted in parallel quantitative decreases in potassium uptake at the period of peak utilization. In this situation, therefore, where potassium was not limiting, the organisms behaved in mixed culture as they did when pure.

Discussion

The potassium uptake of *L. acidophilus* during growth is readily and accurately determinable by the method described. The high reproducibility obtained with the rates of potassium uptake of *L. acidophilus* at the period of high uptake (at which time precision is greatest) permits the technique to be used in evaluating the effects of experimentally imposed conditions. The exact time of peak uptake depends on experimental conditions, but can be readily determined by successive 4 hr sampling.

The rate of potassium uptake of *L. acidophilus* was essentially zero shortly after inoculation, rapidly increased to a maximum between 8 and 16 hr, and then decreased more slowly until by 44 hr it was again zero. Plate counts of cultures at the time of return to zero potassium metabolism showed that the population of viable cells was nearly at the maximum (Shannon & Harris, 1957). Therefore, the cells may be in a state of metabolic dormancy from the time of return of the potassium curve to zero, until eventual extinction of the culture.

Metabolic studies such as these not only delineate small differences in metabolism, but also provide data necessary to gain a better understanding of cellular physiology, and of the rôle of potassium therein, so that correlation of variations in rate of uptake with concurrent changes in morphology, acid production, and cultural characteristics, all of which are of vital importance in studying dental caries formation, becomes feasible.

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THE EFFECT OF SUPPLEMENTATION OF THE GROWTH MEDIUM WITH YEAST EXTRACT, ON DEHYDROGENASE ACTIVITY OF *ESCHERICHIA COLI*

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SUMMARY: Dehydrogenase activities have been determined of washed suspensions of *Escherichia coli* prepared from cultures grown in a synthetic medium, and in the same medium supplemented with yeast extract, under conditions of controlled aeration. This supplementation resulted in increased activity of enzymes attacking L(+)-alanine, DL-serine, formate and succinate, no apparent change in activity of enzymes attacking L(+)-glutamate and D(−)-ribose, and decreased action on D(+)-glucose, mannitol and lactate. The results are discussed in the light of present knowledge of the metabolism of *E. coli*.

During studies on the dehydrogenases of *Escherichia coli*, large cell yields of this organism were required for the preparation of cell-free enzymes; good yields could be achieved by the addition of yeast extract to conventional laboratory culture media, but it was found that on a dry weight basis certain enzymic activities were less than in cells grown in the unsupplemented medium. Experiments reported here aimed at investigating in detail the quantitative variation of selected dehydrogenase activities during the growth cycle of *E. coli* in a simple synthetic medium and in the same medium supplemented with 1% of yeast extract.

The work of Winslow, Walker & Sutermeister (1932) illustrated the fact that *E. coli* grows faster and gives a higher yield of organisms in media containing organic nitrogen in the form of peptones, than in media containing ammonium salts as the chief source of nitrogen, while the marked dependence of the enzyme activity of micro-organisms on the medium used for their growth has been widely investigated. More recent work has resulted in the concept of the fundamental process of enzyme formation as a function of the presence of the inducer, which in the case of constitutive enzymes may be synthesized during the course of cell metabolism (Spiegelman, 1950; Cohn & Monod, 1953) and of the availability of amino-acids for enzyme synthesis. Examples of increased adaptive enzyme production by coli-aerogenes organisms through supplementation of growth media with amino acids have been reported by Pollock & Wainwright (1948), Billen & Lichstein (1951), Pinsky & Stokes (1952) and Pardee & Prestidge (1955). Gary & Bard (1952) and Gary, Klausmeier & Bard (1954) demonstrated the importance of the rôle of the culture medium in determining the metabolic pathway in *Bacillus subtilis*. Cultivation in a tryptone-yeast extract medium yielded cells (C-cells) possessing both the hexose mono- and di-phosphate

pathways of glucose metabolism, and hence capable of both fermentation and respiration, but lacking cytochrome oxidase. Cells grown in an inorganic nitrogen-glucose medium (S-cells) were incapable of glucose fermentation, although possessing the enzymes of both hexose mono- and di-phosphate pathways; these cells contained a vigorous cytochrome oxidase. Enzymic studies revealed that S-cells, as compared with C-cells, contained reduced quantities of glyceraldehyde phosphate and lactate dehydrogenases.

The modification of the metabolism of *E. coli* by additions to the culture medium has been studied by Roberts *et al.* (1955) using radioactive tracer techniques, by which sequences in synthesis have been followed and the quantitative significance of pathways evaluated.

Materials and Methods

Chemicals. Analar reagents were used where available. Sodium lactate was supplied by British Drug Houses Ltd. and complied with the specifications of the B.P. 1932; methylene blue was supplied by Hopkins & Williams Ltd. Ringer's solution was prepared from solution tablets (Oxo Ltd.). The buffer solution used was prepared from KH_2PO_4 and $Na_2HPO_4.12H_2O$, the pH value, 7·0, being checked electrometrically. Bacto yeast extract was supplied by Difco Laboratories Inc., Detroit, Michigan.

Organism. The organism used was *Escherichia coli* NCTC 5934, maintained on slopes of papain digest agar and subcultured weekly. Inocula for experiments were prepared by subculturing on MacConkey's agar for 24 hr, then subculturing typical colonies on the experimental medium for 24 hr; the growth was then washed from the surface with sterile water, the suspension adjusted to standard turbidity and a standard volume used as inoculum.

Growth medium. The medium contained $(NH_4)_2HPO_4$, 0·4%; NaCl, 0·1%; $FeSO_4.7H_2O$, 0·03%; KH_2PO_4, 0·04%; $MgSO_4.7H_2O$, 0·07%; sodium lactate, 1%, or glycerol, 1%. The carbon source was sterilized separately and added aseptically. The medium was supplemented as required with 1% Bacto yeast extract. All concentrations were % w/v. The pH was 7·0.

Method of culture. After sterilization the medium was distributed in 100 ml amounts in tubes 21×3·5 cm, each having a side arm which fitted into a photoelectric nephelometer (Gambrell Bros. Ltd., R.G. Works, Purley Way, Croydon). By tipping some of the culture into the side arm, the extent of growth of the culture could be measured in terms of increase in turbidity at any time. The tubes were placed in a water bath at 37° and aerated for at least 10 min before inoculation.

Aeration. The growth tubes were aerated by negative pressure. The indrawn air was purified by passage through solutions of chromic acid and potassium bicarbonate; it was then passed through water, through a cotton wool filter and by way of a sparger into the culture medium. Routine plating out of cultures failed to reveal any contaminants. A series of cultures could be aerated in parallel, and the rate of flow through each culture tube was controlled, and measured by a separate flow-meter. The antifoam used was 0·14% v/v of a 2% solution of an ethylene oxide derivative of glucose tetrastearate (Crill 27, Croda) in light liquid paraffin BP.

It was found that increasing the rate of aeration from 100 ml/min to 600 ml/min (i.e. from 1 to 6 volumes/min) resulted in increased growth of *E.coli* in lactate media with and without yeast extract. The use of a constant aeration rate, 348 ml/min, enabled reproducible growth curves to be obtained.

Preparation of washed suspensions. Cultures were harvested by centrifugation at 4,500 rev/min for 15 min. The supernatant fluid was poured off, the cells washed once with Ringer's solution, resuspended in fresh Ringer's solution and the suspension standardized to contain 1·92 mg dry weight of organisms/ml, by comparison with a standard suspension of barium sulphate using a photoelectric nephelometer. (Evans Electroselenium Ltd., Harlow, Essex).

Determination of dehydrogenase activity. Dehydrogenase activities of washed suspensions were determined by the Thunberg technique (Umbreit, Burris & Stauffer, 1945). Thunberg tubes were filled as follows:—substrate (0·04 M), 1·0 ml; solution of methylene blue (0·01% w/v), 1·0 ml; phosphate buffer pH 7·0 (0·1 M), 3 ml; Ringer's solution, 0·5 ml; in the cap was placed 0·5 ml of bacterial suspension. After evacuation the tubes were placed in a water bath at 37° and 10 min allowed for equilibration. The contents were then mixed and the colour density was measured at selected time intervals using a photoelectric colorimeter (Gambrell Bros. Ltd., R.G. Works, Purley Way, Croydon) adapted to take Thunberg tubes, and a red filter (Ilford 204).

The time for 90% reduction was estimated by comparison with the galvanometer reading given by a standard tube containing 0·1 ml of methylene blue solution and 0·5 ml of a heat-killed suspension in a final volume of 6 ml. The results were recorded in terms of 'q_{MB}' i.e. 'microlitres' of methylene blue reduced/mg dry weight of cells/hr.

The effect of concentration of organisms

Preliminary experiments showed that suspensions of organisms from an actively dividing culture reduced methylene blue in evacuated Thunberg tubes in the absence of added substrate. This endogenous reduction of the dye was sufficiently marked to complicate the estimation of methylene blue reduction due to dehydrogenation of the added substrate. The endogenous reduction of methylene blue by *E. coli* has been studied by Kopper (1952), who reported that greatest activity was exhibited by cells in the logarithmic phase of growth.

It was found that the contribution of endogenous sources to methylene blue reduction could be minimized by choosing a suitable concentration of organisms. The effect of cell concentration on the q_{MB} values in the presence and in the absence of four representative substrates is shown in Fig. 1; in each case there is a concentration at which endogenous activity disappeared, while activity was still measurable in the presence of added substrate. This effect was also demonstrated in the case of pyruvate and of L(+)-glutamic acid.

The decline in activity of a cell suspension on dilution is attributed to loss of cofactors from the cells by diffusion, so that their effective concentration is below that necessary for certain reactions to proceed. This hypothesis was supported by the observation that the activity due to endogenous substrates, and that due to an added substrate, for example DL-serine, could be restored, after dilution of the

suspension, by the addition of 0·5 ml of the supernatant fluid from a suspension of organisms killed by heating at 98° for 10 min.

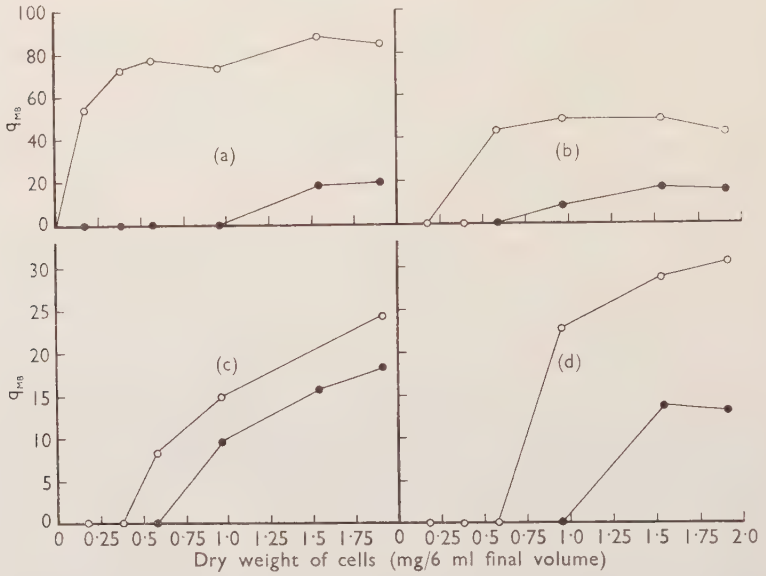

Fig. 1. The effect of concentration of organisms on the endogenous q_{MB} of *E. coli* (full circles) and on the q_{MB} for four substrates (open circles). (a), D(+)-glucose; (b), DL-serine; (c), formate and (d), succinate.

For the main experiments a concentration of 0·96 mg dry weight of organisms in a final volume of 6 ml was selected; this gave a low level of endogenous activity while maintaining enzyme activity in the presence of added substrates. In lactate media, endogenous activity reached a maximum in the logarithmic phase of growth. In both lactate and glycerol media the q_{MB} values were similar in the unsupplemented and in the supplemented media; typical results are included in Figs. 3 and 4.

Results

During each experiment the yield and the dehydrogenase activities of organisms harvested at different stages of growth, were measured. The addition of yeast extract to the culture media resulted in an increased rate of growth and a higher yield of organisms during 24 hr incubation. Typical results are shown in Fig. 2. The graphs show that while with sodium lactate as the main carbon source the pH of the medium increased during growth, the substitution of glycerol resulted in a decrease. In each case the presence of yeast extract appeared to result in a smaller pH shift; this may be associated with decreased utilization of carbohydrate, and with the buffering capacity of the yeast extract.

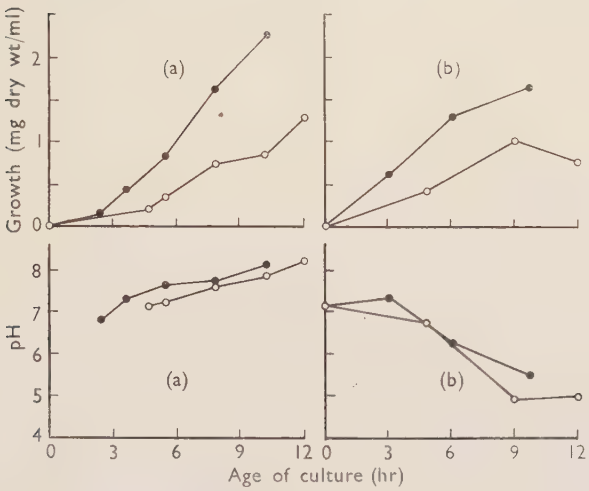

Fig. 2. The influence of supplementation with 1% of yeast extract on the growth and pH changes of cultures of *E. coli* in defined media. Open circles, unsupplemented medium; full circles, supplemented medium. (a), with lactate as the main carbon source; (b), with glycerol as the main carbon source.

The group of substrates chosen for study showed that yeast extract might cause stimulation or inhibition of dehydrogenase activity, or exert no action; this was so when either lactate or glycerol was the main carbon source, and utilization of these substrates was accompanied respectively by an increase or by a decrease of pH. The findings are summarized in Table 1.

Table 1. *The effect of yeast extract on certain dehydrogenases of* E. coli

Dehydrogenase system	Yeast extract effect
L(+)-alanine	Stimulation
DL-serine	
Formate	
Succinate	
L(+)-glutamate	No change
D(−)-ribose	
D(+)-glucose	Inhibition
Mannitol	
Lactate	

The individual results are shown in Figs. 3 and 4, in which the dehydrogenase activities are recorded of cultures harvested at successive stages of growth. For comparison, the total yield of organisms and the endogenous activities are included in representative graphs. In each series, experiments with the unsupplemented and with the supplemented media were performed on different days. During a single experiment a series of growth tubes was inoculated, incubated with controlled

acration, and the organisms harvested at selected stages of growth. Washed suspensions were prepared, stored overnight at 4°, and the dehydrogenase activities determined on the next day.

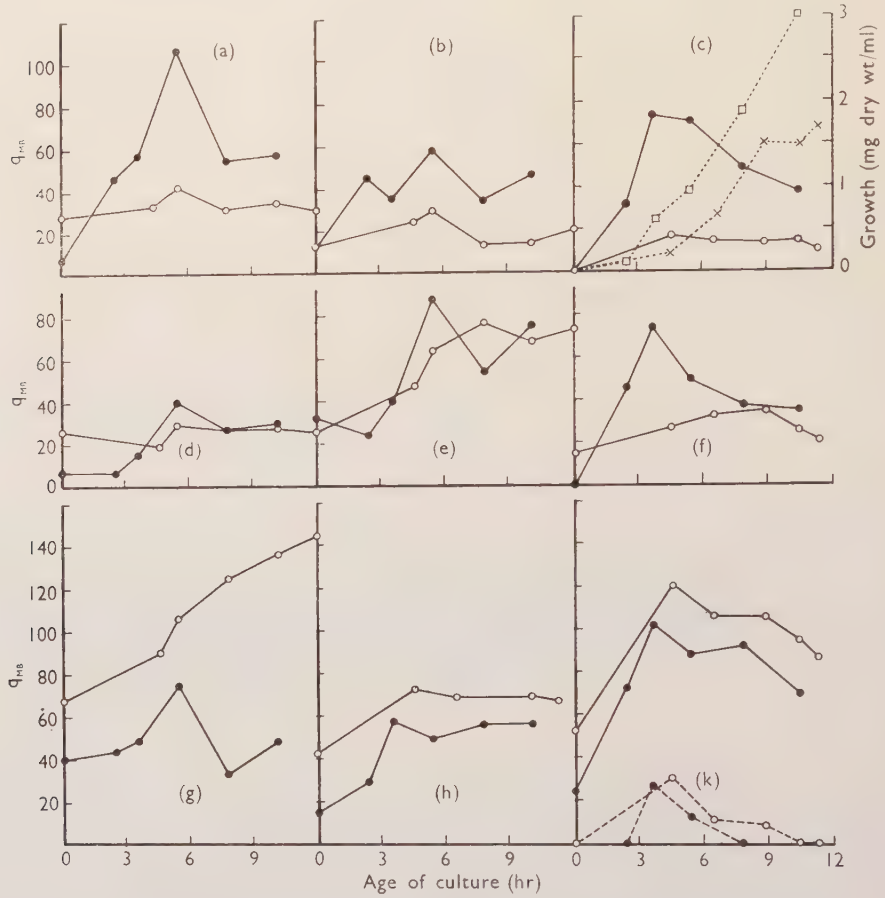

Fig. 3. Dehydrogenase activities (continuous lines) of *E. coli* grown in a lactate-ammonium-salts medium (open circles), and in the same medium supplemented with 1% of yeast extract (full circles). Broken lines, corresponding endogenous dehydrogenase activities in a representative experiment (k).
The growths in the unsupplemented medium (crosses) and in the supplemented medium (open squares) in a representative experiment are also shown (c).
Two series of experiments were done. Series A, with substrates: D(+)-glucose (g), D(−)-ribose (e), L(+)-glutamate (d), formate (b), DL-serine (a); series B, with substrates: L-alanine (c), mannitol (h), succinate (f), lactate (k).

Discussion

The salient point emerging from these results is that when *E. coli* was grown in a medium supplemented with yeast extract, an increase in certain dehydrogenase activities was accompanied by a decrease in others. The increased activity was

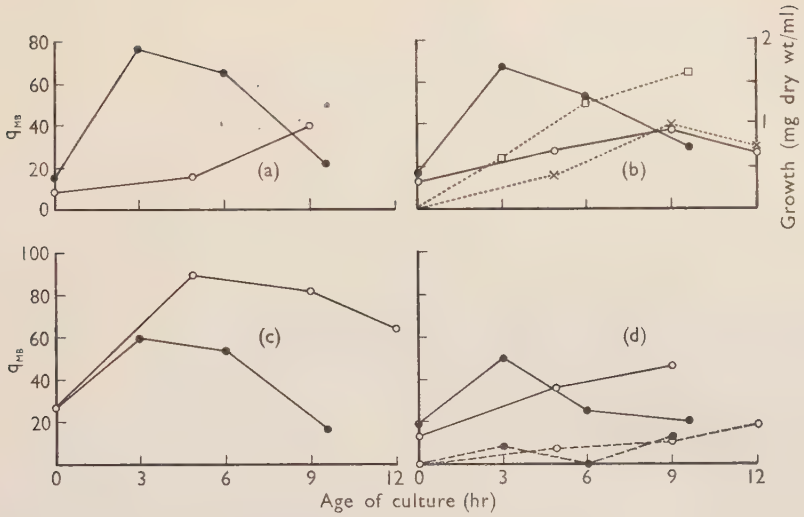

Fig. 4. Dehydrogenase activities (continuous lines) of *E. coli* grown in a glycerol-ammonium-salts medium (open circles), and in the same medium supplemented with 1% of yeast extract (full circles). Broken lines, the corresponding endogenous dehydrogenase activities in a representative experiment (d).
The growths in the unsupplemented medium (crosses), and in the supplemented medium (open squares) in a representative experiment are also shown (b).
(a), DL-serine; (b), formate; (c), D(+)-glucose; (d), L(+)-glutamate.

found in enzymes associated with the Krebs cycle and with amino-acid metabolism, while the decreased activity was associated with enzymes which may be expected to be more directly linked with the Meyerhof-Embden system. The increased activity of formic dehydrogenase when the organism was grown on the supplemented medium is in agreement with the results of Billen & Lichstein (1951), who concluded that while the majority of amino-acids had no effect on this enzyme, glutamic acid, tyrosine, and methionine were all stimulatory.

It is possible that a change in the substrates utilized for metabolism is accompanied by competitive interaction between enzyme systems. Such interactions have been demonstrated by Monod (1947), in his observations on the 'diauxie' occurring in bacterial cultures growing in the presence of a mixture of two or more carbohydrates in limiting concentrations, and by Spiegelman & Dunn (1947) in the case of adaptation to mixtures of maltose and galactose by yeasts. Englesberg, Gibor & Levy (1954*a*, *b*) showed that adaptation of anaerobically grown *Pasteurella pestis* to aerobic conditions was accompanied by induced synthesis of isocitric dehydrogenase, fumarase, cytochrome and free haematin, and decreased activity of phosphofructokinase and glyceraldehyde dehydrogenase.

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THE YEAST FLORA OF APPLE JUICES AND CIDERS

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SUMMARY: The yeast flora of a bulk of apples was followed through the different stages of cider making to the finished product. Yeasts on the fruit, and in the juice from an experimental plant, were mainly asporogenous. In a commercial sized plant, the juice was contaminated with yeasts present on the racks and cloths. Treating the juice with sulphur dioxide improved the yeast flora during fermentation; some controlling factors are considered. The efficiency of centrifugation depended on the pH of the juice, on sulphiting and on the loss of specific gravity that had previously taken place.

Cider has been manufactured for many hundreds of years by allowing juices to ferment naturally, but its yeast microflora has rarely been studied in detail. *Saccharomyces apiculatus*, probably *Kloeckera* spp., was the first yeast to be isolated from apples and juices (Reess, 1870; Hansen, 1881; Boutroux, 1884; Wortmann, 1894). Another yeast, frequently isolated from apples on the Continent, was a top-fermenting torula yeast, able to produce a water soluble red pigment (Kramer, 1891). Subsequently this was called *Torula pulcherrima* (Lindner, 1901), *T. rubefaciens* (Grosbüsch, 1915) and identified as *Candida pulcherrima* (Lodder & Kreger-van Rij, 1952) at the Centraalbureau voor Schimmelcultures (C.B.S.). The pigment (van der Walt, 1952; Cook & Slater, 1956) was erroneously considered to be water soluble by the earlier workers because it diffused readily into the agar medium.

Kayser (1890) and Dienert (1896) in France, and Lloyd (1903), Pearce & Barker (1908) and Barker (1950, 1951) in England, isolated yeasts from high quality ciders, but the cultures cannot be identified today from their published descriptions.

Undoubtedly the best work was done by the Swiss workers, Osterwalder (1912, 1924 and 1934) and Müller-Thurgau (1903). *Monilia vini* was isolated from the lees of an apple wine; it was renamed *Oospora* (*Monilia*) *vini* Osterwalder (Janke) and later grouped with *Mycotorula intermedia* (Krumbholz & Tauschanoff, 1933) as *Brettanomyces bruxellensis* (Diddens & Lodder, 1942). This is the only record of a species of this genus being isolated from apples or their products. Osterwalder described *Saccharomyces microellipsodes* from apple pulp, *S. heterogenicus* from apple juice, *S. globosus* (*S. delbreuckii* var. *mongolicus*) from pear juice, and *S. tubiformis* (*S. willianus*) and *S. intermedius* Hansen var. *turicensis* (*S. uvarum*) from naturally fermenting pear juices.

Capitain (1930) isolated twenty-two yeasts from pear wines; eleven were later identified at the C.B.S. as *S. uvarum*, six as *S. cerevisiae*, three as *S. cerevisiae* var. *ellipsoideus*, one as *S. willianus* and one as *S. acidifaciens*.

Recently Clark, Wallace & David (1954) and Williams, Wallace & Clark (1956) isolated yeasts from wild and cultivated Canadian apples; they identified *Candida*

malicola (*Pullularia pullulans*), *C. scottii*, *C. krusei*, *Torulopsis famata*, *T. candida*, *Kloeckera apiculata*, *Cryptococcus albidus*, *Cr. neoformans*, *Cr. laurentii*, *Rhodotorula glutinis* var. *rubescens*, *R. mucilaginosa* and *S. bisporus*. Clark *et al.* (1954) noted that two barrels of dry cider contained *Pichia membranaefaciens*, *P. polymorpha*, *Debaryomyces kloeckeri*, *S. oviformis*, *S. cerevisiae*, *C. steineri*, *C. mesenterica* and *T. candida*. The ciders must have been exposed to air during storage to contain such a high proportion of film yeasts.

Apart from these detailed studies, yeasts have been isolated from spoiled fruit, juice or apple products, i.e. *Endomyces mali* (Lewis, 1910), *S. rouxii* and its variety *polymorphus* (Naganishi, 1928), *S. elegans* and *C. krusei* (Lodder & Kreger-van Rij, 1952).

It was impossible to tell from the literature whether cider was fermented by yeasts derived from the fruit or from the milling and pressing equipment. This was the object of the work reported below and it was undertaken by examining the yeast flora of the fruit and through all the cider making processes to the final cider.

Experimental Methods

The methods used for sampling, counting, isolating and identifying the components of the yeast flora have been reported elsewhere (Beech, 1956*a*, 1957; Beech & Carr, 1954, 1955, 1957, 1958; Beech, Carr & Codner, 1955).

In any ecological study of yeasts, the number of species isolated will depend on the proportion each forms of the total population of the sample. Thus, if a yeast forms less than 2% of the flora there must be at least 100 colonies on the isolation plate and this is not always possible to arrange since the number of viable yeasts present cannot be determined before plating out. It is for this reason that yeasts present in small amounts cannot always be found in successive samples. If subsequent processing of the cider removes the major component of the yeast flora, the minor components are more likely to be isolated, since they will then form a larger proportion of the flora. It has subsequently become possible to count small numbers of yeasts other than *Saccharomyces* spp. in the presence of overwhelming numbers of yeasts of the latter genus (Morris & Eddy, 1957). The converse, direct counting of small numbers of *Saccharomyces* spp., is not yet possible.

Most of the results described were obtained after processing a large bulk of apples of the variety Kingston Black; this yielded a juice of specific gravity 1,060°, pH 3·45 and a low nitrogen content, 3·2 mg N_2/100 ml. Where relevant, results with juices of higher and lower pH are also quoted. Isolates not described previously in the literature have been given a code letter.

Results

Yeast flora of the fruit

The flora present on the skin, at the bases of the stalks and calyces and in the cores were examined separately; the results are given in Table 1. Apart from the flesh, which was not examined, yeasts were present in all parts of the apple. Washing the fruit removed only a proportion of the yeast flora: the theory, held by some cider

makers, that fruit washing removes all the yeasts does not appear to be justified. *S. uvarum* was the only isolate of this genus from the fruit; normally enrichment techniques are necessary to demonstrate their presence on sound fruit. Species of the genus *Hansenula* have not been isolated previously from apples; a number of the *Candida* spp. assimilated nitrate and may prove to be haploid forms of *Hansenula*. *C. pulcherrima*, a yeast forming maroon-coloured colonies, was isolated from most parts of these apples and those of other apple varieties examined at Long Ashton. Surprisingly, in view of the statements of Clark *et al.* (1954), Williams *et al.* (1956) and Marshall & Walkley (1952), no carotenoid-forming yeasts were found on the fruit. Only two species of the genus *Rhodotorula* have been found among 1,400 isolates from apples, juices and ciders at Long Ashton; one, *R. mucilaginosa*, was found in the water used for washing the fruit before it was processed on the pilot scale plant.

Table 1. *Yeasts isolated from Kingston Black fruit*

Yeast	Washed fruit				Unwashed fruit		
	Eyes	Cores	Stalks	Skin	Eyes*	Stalks*	Skin
Saccharomyces uvarum							1%
Hansenula B	1 col.†	20%		44%		7½%	
Pichia fermentans						1 col.	2%
Debaryomyces kloeckeri							50%
D. nicotianae					20%	8%	
Candida A						1 col.	
Candida B		2 cols.					
Candida D						2½%	
Candida F	40%	75%		1 col.			1%
C. pulcherrima	1 col.	4%	1%		25%	1 col.	35%
C. parapsilosis var. *intermedia*			9%	6%			
C. catenulata				50%			
C. solani						1 col.	
Torulopsis A		1 col.	90%				
Torulopsis C	60%			1 col.			
Died after isolation	5%	2 cols.					12% Butyrous

* Remainder of the flora not examined. † col., colony.

Juice flora

The yeast flora of the juices, obtained by processing portions of the same bulk of fruit on a pilot and a commercial scale plant, is given in Table 2. There is a remarkable difference in the composition of the flora of the two juices. That from the pilot plant, which could be cleaned satisfactorily, was very similar to the fruit flora, consisting mainly of asporogenous yeasts. From the commercial scale plant, in which the racks and cloths were not cleaned frequently, the flora consisted almost entirely of *Saccharomyces* spp. Two of these yeasts, *S. microellipsodes* and *S. cerevisiae*, were isolated only from juices or ciders processed on this commercial plant and must, therefore, be considered as being the 'factory flora'. The infection probably resided in the racks and cloths, as observed also by Marshall & Walkley (1951) in their survey of a commercial apple juice plant. The same effect is also shown by a comparison of viable counts on juices from other batches of fruit, processed on both the

pilot and commercial scale plants (Table 3). The contamination of juice in cider factories can be very deleterious if the added yeasts have poor powers of fermentation, or the bacteria are rope-forming lactobacilli (Beech & Carr, 1953).

Table 2. *A comparison of the yeast flora of Kingston Black juice from pilot and commercial scale plant*

Yeast	Pilot scale	Commercial scale
Candida pulcherrima	53%	2·25%
Candida F	10%	12%
Cryptococcus neoformans	3%	
Unknown 2	3%	
Rhodotorula minuta	3%	
Torulopsis A	12%	0·5%
Apiculate yeasts	3%	
Dead yeasts	13%	17%
Pichia fermentans		0·25%
Hanseniaspora osmophila		7%
Saccharomyces uvarum		1·50%
S. cerevisiae II		7%
S. microellipsodes		52·5%

Table 3. *The numbers of viable yeasts and bacteria in juices from samples of the same fruit pressed on pilot and commercial scale plants*

	Yeast counts (10^3/ml) in juices from					
	Kingston Black		Bramley's Seedling		Sweet Coppin	
	Pilot scale	Commercial	Pilot scale	Commercial	Pilot scale	Commercial
Yeasts	300	1,055	477	665	610	1,372
Bacteria	—	—	17	139	135	185

The effect of sulphur dioxide on the juice flora

From earlier work (Beech 1953, 1956*b*; Beech, Burroughs & Whiting, 1956) it was known that addition of sulphur dioxide to the fresh juice had a beneficial effect on the subsequent fermentation. Sulphur dioxide does not act immediately. The best time for sampling was found by adding pure cultures of *S. uvarum* and *C. pulcherrima* to a citrate buffer at pH 3·0 containing 1·8% of glucose and 15 and 25 p/m of SO_2 respectively; the glucose concentration was the same as is found in juices, and the solutions were allowed to equilibrate for 24 hr before inoculation. There was little reduction in the numbers of *S. uvarum* after 5 hr, but *C. pulcherrima* was destroyed after 3 hr in the solution. *S. uvarum* was then added to sterile juices of different pH containing 150 p/m of SO_2. That concentration at pH 4 is equivalent to 15 p/m at pH 3·0 (Vas & Ingram, 1949). The results are shown in Table 4. In the presence of the juice nutrients the yeast began to grow after a lag phase, which was 5 hr at pH 4·0 and increased with decreasing pH. Subsequently, juices were sampled 4 hr after adding the sulphur dioxide, and Table 5 shows the effect of different concentrations on a fresh juice of pH 4·0. The amount added in practice is varied according

to the pH of the juice; 150 p/m at pH 4·0 and above, 100 p/m between pH 3·4 and 4·0 and 50 p/m below pH 3·4.

Table 4. *The effect of SO_2 (150 p/m) on* Saccharomyces uvarum *in sterile apple juices of different pH*

Time of contact (hr)	Viable count/ml at pH		
	3·4	3·7	4·0
0	3,500	850	5,300
1	2,000	800	5,150
2	1,880	800	5,150
5	650	880	6,500
24	N.S.	35,000	>10,000
168	10,000,000	N.S.	N.S.

N.S., not sampled.

Table 5. *The effect of different concentrations of sulphur dioxide on the numbers of viable yeasts and bacteria in Sweet Coppin juice of pH* 4·0

Concentration of SO_2 (p/m)	Viable count/ml after 4 hr	
	Yeasts	Bacteria
None	610,000	135,000
100	65,000	<4,500
200	4,800	<600
250	2,500	<500

When 150 p/m were added to a sample of the Kingston Black juice (pH 3·45) pressed out on the pilot scale plant, the numbers of yeasts were reduced to 1·5% of those present in the untreated juice. The principal surviving yeasts, all capable of fermenting glucose, were *Pichia fermentans*, *Torulopsis* spp. A and C and *C. pulcherrima*.

Changes in the microflora during fermentation

The composition of the microfloras of the sulphited and untreated pilot scale Kingston Black juices during fermentation are given in Table 6. In the untreated juice, most of the yeasts died out between 0° and 5° loss in sp. gr. At the same time *S. uvarum*, present originally on the fruit, increased from a minute proportion of the flora to become the dominant yeast. Maximum growth of this yeast occurred between 10° and 20° loss in sp. gr. and the viable count decreased continuously thereafter. Apiculate yeasts increased in numbers between 0° and 5° loss in sp. gr., then declined and finally disappeared after 35° loss. About this period there was an increase in the numbers of a yeast, called at present Unknown 1, and bacteria. The extent of bacterial development is largely dependent on the original pH of the juice, increasing with increasing pH. With juices of high pH, a malo-lactic fermentation due to bacteria can occur before yeast fermentation begins (Carr & Whiting, 1956).

In the sulphited juice fermentation, all yeasts except *S. uvarum* died out between 0° and 5° loss in sp. gr. *S. uvarum* showed the same type of growth curve as in the untreated juice but there were nearly 70% more viable yeast cells present during

Table 6. *The effect of SO_2 (150 p/m) on the changes in the composition of the microflora of Kingston Black juices during fermentation (pilot scale)*

Loss of specific gravity*	Numbers of yeasts and bacteria (10^3/ml) in Non-sulphited juice				Sulphited juice	
	S. uvarum	Apiculate	Unknown 1	Bacteria	*S. uvarum*	Unknown 1
5°	11,650	880	—	—	17,600	—
15°	22,400	250	—	—	37,500	—
25°	12,350	180	1,150	50	25,100	—
35°	7,280	100	2,750	150	6,250	—
45°	5,800	—	1,500	180	3,850	<50
55°	5,000	—	900	300	2,640	360

* Initial specific gravity, 1,060°

Table 7. *The effect of juice sulphiting (150/p/m) on the ultimate microbial composition of dry ciders made from juices of different pH*

Organisms	Numbers of viable organisms after fermentation (10^6/ml) Bramley's Seedling pH 3·2		Kingston Black pH 3·5		Sweet Coppin pH 3·9	
	No SO_2	SO_2	No SO_2	SO_2	No SO_2	SO_2
Yeasts	50*	53·5	8·5	60	10	60
Bacteria	N.D.†	N.D.	57	N.D.	3	N.D.

* Many apiculate yeasts. † N.D., not detected.

the period of maximum development, probably due to lack of competition for the juice nutrients. For the greater part of the fermentation there was a pure culture of *S. uvarum* but towards the end a slight development of Unknown 1 occurred: bacteria were not detected at any stage of the fermentation. The beneficial effect of juice sulphiting can also be tested by examining the microflora of dry cider; results for three dry ciders, made from juices of pH 3·2, 3·5 and 3·9 respectively, are given in Table 7. Carr & Whiting (1956) followed bacterial development in fermenting juices by chromatographic analysis of the organic acids; their results are summarised in Table 8. The titratable acidities of untreated juices usually decrease during fermentation, whereas in sulphited juices they increase.

Table 8. *The effect of pH and sulphur dioxide on the activity of lactic acid bacteria during fermentation*

(Data from Carr & Whiting, 1956)

pH of original juice	Changes occurring in Untreated juice	Sulphited juice
<3·4	Malo-lactic fermentation	No change in acidity
3·4–4·0	Malo-lactic or malo-succinic change. Loss of acidity offset by	Increase in malic acid due to yeasts
>4·0	formation of lactic acid from sugar	Possibility of malo-lactic change, acidity loss offset by formation of lactic or succinic acid from sugar

The effect of centrifugation and fermentation on the microflora of juices

Rapidly fermenting juices are often centrifuged in the early stages of fermentation in order to control the rate of fermentation. Burroughs (1953) has shown from changes in the soluble nitrogen content that this acts via a removal of yeasts whose growth has depleted the cider of amino acids and thiamine. To determine the effect of the treatment on the components of the yeast flora, barrels of the Kingston Black juice from the commercial scale press were allowed to ferment for different periods and sampled before and after centrifuging. The results are given in Table 9, the yeasts having been grouped according to genera to restrict the size of the Table.

Table 9. *The effect of centrifuging on the microflora of fermenting Kingston Black juices*

Decrease in specfic gravity	Condition in regard to centrifuging	Numbers of different organisms (10^3/ml)			
		Saccharomyces and *Hansenula* spp.	*Torulopsis* and *Candida* spp.	Apiculate yeasts	Bacteria
5°	Before	3,894	—	2,006	—
	After	43	86	1,940	55
10°	Before	1,742	307	31	50
	After	23	—	—	35

Centrifuging after only 5° loss in sp. gr. was obviously undesirable; the bacteria and smaller yeasts passed through the centrifuge virtually unchanged in numbers. This yielded a centrifugate with an undesirable flora since apiculate yeasts are known to produce unwanted volatile acids in cider (Müller-Thurgau, 1899). Centrifuging after 10° loss in sp. gr. gave a centrifugate containing mainly *Saccharomyces* spp. that are better for continuing the fermentation. Apiculate yeasts were no longer dangerous

Table 10. *The effect of clarification treatments on the numbers of yeasts and bacteria in dry ciders made from juices of low and high pH with and without SO_2*

Treatment	Viable counts (10_3/ml) in							
	Bramley's Seedling (low pH)				Sweet Coppin (high pH)			
	Untreated		Sulphited		Untreated		Sulphited	
	Yeasts	Bact.	Yeasts	Bact.	Yeasts	Bact.	Yeasts	Bact.
Mixed	29,000		55,000	N.D.*	600	10,350	27,000	+
Centrifuged	1·8	8·05	0·95	N.D.	0·04	4,300	<1	N.D.
Centrifuged and filtered	N.D.	0·12	N.D.	N.D.	N.D.	8·65	N.D.	N.D.

*N.D., not detected.

as their numbers had decreased considerably at this stage of the fermentation. An undesirable feature of this second centrifugate was the change in the ratio of yeasts to bacteria. From the results obtained with pilot scale juices it was apparent that it would be better to centrifuge ciders made from sulphited juices. Table 10 shows the

effect of clarification treatments on dry ciders made from juices of low and high pH. In the cider from Bramley's Seedling juice, the bacterial growth was prevented by the low pH. Sulphiting caused greater growth of yeasts in both ciders. The numbers of viable yeasts at the end of the fermentation is dependent on the nutrient status of the juice. The Kingston Black juice in Table 7 was low in nutrients and the yeast count was low in the dry cider; the juices in Table 10 had a higher nitrogen content originally and a much higher yeast count finally. In the Sweet Coppin cider (high pH), there was an enormous bacterial population in the untreated sample which, after centrifuging, overwhelmed the yeast flora. The contrast between the flora of this cider and its sulphited partner was most marked. Filtration following centrifugation is obviously desirable, but this cannot remove taints produced by the prior growth of bacteria in ciders from untreated juices.

Discussion

There is still a great deal to be learnt about the changes that take place in the microflora of apple juices and ciders. Nothing has been said of the minor components of the yeast flora, i.e. yeasts forming less than 1% of the population during fermentation. It is known that among these are *Brettanomyces* spp. and *Saccharomycodes ludwigii*. The latter is capable of causing serious economic losses in the industry if conditions favour its development, while *B. anomalous* has been isolated from a dry cider with an enrichment technique. Wooden fermentation vessels, used widely in cider making, often have yeasts and sometimes bacteria in their timbers and these can affect the fermentations. The yeast floras of orchards in different counties and in other factories need investigation, since recent observations suggest that different species of the genus *Saccharomyces* can be found. In some factories the main fermenting yeast is circular and non-flocculating and, although it ferments strongly, it makes filtration of the final ciders more expensive.

With these provisos the present investigations have shown that improvements can be introduced into cider making. One result has been the practice of sulphiting the fresh juice, a method that has now been adopted in most cider factories over the last four years with great improvement in the vintage quality of the ciders. The method used for clarifying ciders can now be chosen according to the pH, nutritional status and the pretreatment given to the juice.

Other advantages are beginning to be apparent from an examination of the special properties of the individual isolates. Strongly fermenting bottom yeasts can be chosen that are sulphite tolerant and are not exacting in their nitrogen and vitamin requirements. Other isolates have been shown to degrade pectic acid with polygalacturonase (Pollard & Kieser, 1959), which in combination with the pectinmethylesterase from the fruit removes pectin from ciders during fermentation. Other yeasts synthesize malic acid in fermenting sulphited juices of medium to high pH (Beech & Pollard, 1956; Beech, Burroughs & Whiting, 1956). The activity of such yeasts reduces the cider maker's need for sharp varieties of cider apples and for citric acid, formerly used to restore loss of acidity by malolactic fermentation in non-sulphited ciders.

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LACTIC ACID BACTERIA AS SPOILAGE ORGANISMS OF FRUIT JUICE PRODUCTS

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SUMMARY: The spoilage of wines and ciders by lactic acid bacteria is described. The pioneering work on wine bacteriology at the turn of the century is discussed in addition to modern work carried out in Australia and the U.S.A. Similarly, past and present work on the spoilage of ciders by lactic acid bacteria is discussed, special reference being made to their activity against malic acid as well as chlorogenic acid and its derivatives.

The scope of this paper will be limited to spoilage in two fruit juice products, namely wines and ciders, in order that they may be discussed in some detail. The study of the microbiology of fermented fruit juices really started with the publication of *Étude sur le Vin* by Pasteur in 1866. From that time until the end of the century a large number of chemical and microbiological observations were recorded on wine without very much attempt being made to correlate them. It was not until the 1900's that a clearer picture of microbial activity in wines began to emerge.

The early work on wines

The two people who made the most outstanding contributions to this topic were Müller-Thurgau & Osterwalder (1913), working in Switzerland. They showed, for example, that the much discussed production of mannitol in wines could be caused by at least four species of bacteria (Müller-Thurgau & Osterwalder, 1918) and it thus became clear that the 'ferment mannitique' observed by other workers previously was due to the bacterial reduction of fructose. The best known of these bacteria was one described by these workers as *Bacterium mannitopoeum*, more familiarly known today as *Lactobacillus buchneri*. They showed that, in addition to producing mannitol from fructose, these heterofermentative organisms form lactic and volatile acids from several sugars, including fructose, giving rise to taints. They showed further that a number of bacteria, both rods and cocci, were able to break down malic acid to lactic acid in fermented grape, apple and pear juices, thus reducing the acidity. Further, they stated that rod shaped organisms taking part in this so-called malo-lactic fermentation caused taints, while cocci under similar circumstances led to an amelioration of the acidity without taint. The reason for this would seem to be that the rods are faster growing organisms than the cocci and therefore better able to compete with the yeasts during the later stages of fermentation and thus produce undesirable end products from the available sugars. The cocci, on the other hand, would tend to be suppressed by yeast fermentation and only become active when the source of the taints, namely sugars, had been fermented away.

The most widely discussed malo-lactic coccus is one called *Bacterium gracile*, a name which is still widely used in Swiss and German wine and fruit juice literature.

According to Bergey's Manual, (Breed, Murray & Hitchens, 1948) this is probably not a *Lactobacillus* but a *Leuconostoc* species. Pederson (1952) stated as his opinion that *B. gracile* should be regarded as a non-slime-forming strain of *Leuc. mesenteroides*. Having examined an authentic culture of this organism from Switzerland I would agree with this view.

In addition to the bacteria already mentioned, Müller-Thurgau & Osterwalder (1919) were able to show that a heterofermentative lactobacillus called *Bacterium tartarophthorum* decomposed tartrate and gave rise to acetic acid plus CO_2. The same organism could also decompose glycerol, producing acetic, propionic and lactic acids.

The later work on wines

More recently Lüthi (1953), working at the same place and in the tradition of Müller-Thurgau & Osterwalder, isolated and described a coccus which was responsible for the disorder known as ropiness in wines. This he called *Streptococcus mucilaginosus* var. *vini*, which implied it was similar to a slime forming organism found in beer. It is stated that this organism is heterofermentative; which creates a paradoxical situation since by definition members of the genus *Streptococcus* are homofermentative, so that this organism is either not a *Streptococcus* species or not heterofermentative.

Other recent work on wine bacteria has been carried out by Fornachon (1943) in Australia. He studied organisms causing spoilage in fortified wines and found that they could be divided into five groups. Of these, one had not been described previously while the others either resembled *L. buchneri* or *L. fermenti*. Fornachon later joined forces with Vaughn and others in California, where they added to the existing names by describing a heterofermentative rod shaped organism which they named *Lactobacillus trichodes*, because of its characteristic formation of twisted masses of filaments (Fornachon, Douglas & Vaughn, 1949). This, plus its ability to cause spoilage in the presence of 20% (v/v) ethanol were its two outstanding characteristics. This team also redefined the species *L. hilgardii* (Vaughn, Douglas & Fornachon, 1949) which caused spoilage of Californian wines. They suggested that this organism, *L. trichodes*, *L. fructivorans* and *Betabacterium caucasicum* (renamed *L. desidosus*) should all be considered to belong to an inactive group, being distinguished from other *Lactobacillus* spp. by growing slowly even under optimum conditions, taking a minimum of 4–5 days and sometimes up to 14 days to develop visible growth.

Thus, spoilage of wines by lactic acid bacteria takes the following forms. 1, The production of excess acidity from sugars, due to (a) homofermentative bacteria producing lactic acid and (b) heterofermentative organisms forming lactic acid, acetic acid and CO_2, and mannitol from fructose; 2, the same excess acidity, accompanied by a mousey flavour (acetamide); 3, the decomposition of tartaric acid, giving acetic acid and CO_2; 4, the decomposition of glycerol, with the production of acetic, lactic and propionic acids, and 5, the decomposition of malic acid giving lactic acid and CO_2. The last is not generally considered as spoilage.

Past work on cider

The very early work on ciders was carried out by the Swiss and there were some tentative attempts to examine lactic acid bacteria in this country at the turn of the

century. However, the most intensive work was by Millis (1951), who studied the disorder of ropiness or oiliness which can cause great economic loss to the cider maker. This type of spoilage, which results in a general thickening of the beverage, can be caused either by a rod shaped organism or a coccus. Of the two, the former is the more prevalent and Millis found that it was a heterofermentative species which she considered to be related to *L. brevis*. In pure culture this organism made slime from glucose, fructose and maltose. The first two of these sugars are abundantly present in apple juices and sweet ciders while the third is never found at all. None of the slime forming strains utilized sucrose for polysaccharide synthesis; therefore, the pattern of slime formation is quite unlike that found for *Leuconostoc* spp.

The slime forming coccus was identified as a *Leuconostoc* sp., possibly *mesenteroides* but differing from it by not being able to build slime from sucrose. This particular coccus must be rare because Millis only isolated a single strain, and all ropy ciders investigated since have been caused by the rod shaped organism. Unfortunately, this coccus has now ceased to produce slime.

Present work on cider

More recently I have been concerned with the lactic acid bacteria that occur in ciders, irrespective of whether they cause apparent spoilage or not. During this investigation it was found that the two commonest organisms were a heterofermentative rod which was considered to be a strain of *L. pastorianus* (Carr, 1957) and a heterofermentative coccus. The former is perhaps the most common organism in cider and its properties have been studied in some detail. Like its wine counterparts it will attack sugars and produce lactic and acetic acids and CO_2. In addition it will reduce fructose to mannitol. This organism is almost identical with the slime forming organism save for the production of polysaccharide. One slime forming rod gradually lost this capacity over a long period of sub-culturing and then became indistinguishable from the strains that had never shown slime formation.

Perhaps the most interesting activities of this organism are those involving the organic acids of ciders, in which the main acid is malic with quinic the next most abundant. As mentioned previously, the wine bacteria are capable of attacking malic acid and converting it to lactic acid. This is also true of *L. pastorianus*, which converts the malic acid in part to lactic acid and in part to succinic acid. At pH 3·2, lactic acid is the principal acid, succinic acid only being present as a trace. As the pH increases to 4·4 the amounts of the two end products become equal. If these organisms are grown at pH levels above those normally encountered in ciders, e.g. 4·8, the lactic acid diminishes and the succinic acid increases. However, over the range of normal juice pH values (3·2 –4·2) there is always a mixture of two acids from the decomposition of malic acid.

One unexpected activity of *L. pastorianus* is its ability to convert quinic to dihydroshikimic acid, with shikimic acid probably involved as an intermediate (Carr *et al.* 1957). The possible participation of shikimic acid may be of great importance because Davis (1955) showed it to be the key acid in the synthesis of various aromatic amino acids. Further work with this strain of *L. pastorianus* has shown that it is able to hydrolyse chlorogenic acid into its two components, caffeic and quinic acids.

The fate of quinic acid has already been discussed, while caffeic acid is first reduced to dihydrocaffeic acid and subsequently decarboxylated, forming ethyl catechol (Whiting & Carr, 1957). This organism thus possesses a most unusual mechanism, which could possibly be involved in aromatic amino-acid synthesis or, perhaps alternatively, in the utilization of these various organic acids simply as unusual hydrogen acceptors.

This organism can also attack citric acid with the production of lactic and acetic acids. This is not of importance in apple juices since citric acid occurs only in traces, but it may be important in pear juices where it is sometimes found in amounts equal to those of malic acid. It may also be of importance in a low acid cider to which citric acid has been added to adjust the acidity.

The heterofermentative coccus mentioned before is a non-slime-forming strain of *Leuc. mesenteroides*. The fact that these organisms, *B. gracile* and some *Leuconostoc* spp. described by Pederson & Albury (1955) do not produce slime from sucrose suggests that this characteristic may be less widespread among freshly isolated strains of *Leuc. mesenteroides* than has hitherto been claimed. Most of the activities of the cocci are similar to those of *L. pastorianus* except that quinic and related acids are not metabolised and the range of sugars attacked is different. On the whole the metabolic processes of the cocci proceed much less rapidly than those of the rods.

In addition to the heterofermentative organisms, a few homofermentative rods (but no cocci) have been found. Most of these appear to be anomalous strains and it has been possible to identify one culture only, as a strain of *L. plantarum*. Among these organisms one strain was discovered which is catalase positive, thus adding to the small number of such lactobacilli which have already been described (Dacre & Sharpe, 1956; Vankova, 1957). These organisms also produce lactic acid from sugars but, unlike the heterofermenters, they do not produce other end products as well. Likewise, they are able to carry out a malo-lactic fermentation, but their activities against other acids are, on the whole, more limited than those of the heterofermenters.

Thus, the lactic acid bacteria of ciders are able to carry out most of the chemical activities of their counterparts in wine. Indeed, their activities may cover a wider range than those of the wine bacteria. However, inoculation of a few selected non-slime-forming strains into cider has shown that none of these organisms produces anything as objectionable as the mousey taint of wines. In fact, so far as the flavour is concerned, the only evidence of bacterial activity is a loss of fruitiness and lessening of the acidity due to the destruction of malic acid. From this limited evidence it can only be concluded that the relatively poorer nutritional status of apple juice for bacteria compared with that of grape juice is a major factor contributing to this difference.

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A SURFACE FILM METHOD OF EVALUATING QUATERNARY AMMONIUM COMPOUNDS

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SUMMARY: A suspension of bacteria was evenly spread on descaled stainless steel strips and air dried. The strips were put into the QAC solution and after the required period the bactericidal action was stopped by adding a neutralizing agent. The strips were covered with nutrient agar and incubated at 37° for 24 hr to enable the surviving organisms to grow into colonies. On removing the thin layers of agar from the strips the colonies were left adhering to the metal. They were stained with carbol fuchsin and counted using a dissecting microscope.

Using the recommended strength of a QAC-detergent solution (200 p/m QAC) and an exposure time of 2 min a mortality of at least 99·58% was obtained. The temperature of treatment had little effect on this, though in experiments with added organic matter or lower concentrations of QAC the temperature was important.

The bactericidal power of a solution is usually officially estimated by the Rideal-Walker test (Specification, 1934) or by the F.D.A. test (Association of Official Agricultural Chemists, 1950). These methods are generally recognized as being unreliable and misleading with compounds of the quaternary ammonium group (Mallman & Hanes, 1945; Quisno, Gibby & Foter, 1946; Klarmann & Wright, 1946; Johns, 1947). During the last fifteen years much work has been done to find a satisfactory laboratory method of evaluating QACs and two lines of investigation have been followed. First, there are the adaptations of the official methods already mentioned which involve inoculating organisms into the antibacterial solution (Weber & Black, 1948; Davies, 1949; Cousins, 1951). Second, there are the methods in which the antibacterial solution acts upon a film of organisms adhering to a surface (Mallman & Hanes, 1945; Klarmann & Wright, 1946; Johns, 1947; Neave & Hoy, 1947; Stuart, 1947; Garvie & Clarke, 1955; Hirsch & Muras, 1955). The surface film technique used by Garvie & Clarke (1955) formed the basis for the present experiments. Because of its adaptation for use with or without organic matter in the surface film a suitable metal surface had to be standardized and a new method of evaluating the surviving organisms developed.

Materials and Methods

A loopful of bacterial suspension was spread evenly over a standardized metal surface and air dried. This contaminated strip was soaked in the QAC test solution. At the end of the required exposure period neutralizing agent was added and the two solutions mixed by a gentle rotary movement. After a further 5 min soaking to allow the neutralizer to be fully effective the strip was removed and covered with nutrient agar in a Petri dish. The dish and strip were incubated at 37° for 24 hr before the covering agar layer was peeled off, and the exposed colonies on the strip

were then stained with carbol fuchsin. Enumeration of the colonies was done with the aid of a cellophane graticule and a dissecting microscope.

Sanitizing test solution. The solution tested was the QAC-detergent compound Lactosan '66' (Gascoigne-Crowther Ltd., Reading) made up in tap water. The active constituent of Lactosan '66' is alkyldimethylbenzylammonium chloride and subsequent dilutions were made on the basis of this constituent. Using the test of Harper, Elliker & Moseley (1948) the concentration of QAC in a solution of Lactosan '66' prepared according to the manufacturer's directions was found to be 202·5 p/m. The hardness of the tap water used was constant at 3° Clarkes.

No attempt was made to control the pH of the test solutions; the values ranged from 10·55 at 50 p/m to 10·9 at 200 p/m.

Test culture. Staphylococcus aureus (Burroughs Wellcome CN 491) was used throughout. The stock culture was maintained on nutrient agar (Oxoid CM 3) at 4° and was transferred to a fresh slope every 2–4 months.

Preparation of bacterial suspension. Subcultures were made on nutrient agar slopes and incubated at 37° for 24 hr. It had previously been found that only within the range of the 3rd to the 7th transfers on agar was the resistance of the culture to adverse conditions reasonably constant (Whitt, 1958). The bacterial suspension was made by scraping a small amount of growth from such a slope and transferring it to a McCartney bottle containing 9 ml of quarter-strength Ringer's solution and glass beads. In one experiment 10% of milk was present. Shaking for 4 min produced a suspension from which a reproducible loopful could be withdrawn, the number of organisms in a loopful varying between 187×10^3 and 472×10^3 over the 15 experiments.

Treatment of metal strips. To facilitate even spreading of the bacterial suspension over the metal, without added organic matter being present, it was necessary to modify the stainless steel surface; descaling proved successful and Staybrite F.S.L. Steel, supplied already descaled by Firth-Vickers Ltd., Sheffield, was used. The strips measured $2 \times 0{\cdot}5 \times 0{\cdot}064$ in. and were soaked in sour milk for two days before use to remove any taints in the metal (Neave & Hoy, 1947; Garvie & Clarke, 1955). For complete evenness of spreading it was necessary to scrub the strips in detergent, rinse thoroughly, autoclave to sterilize, and finally soak in absolute alcohol for several hours; after flaming and cooling the strips were ready for inoculation.

Inoculation of the strips. A loopful of suspension was smeared evenly over the strip covering a central area of approximately $1\frac{1}{4} \times \frac{1}{4}$ in. On the evidence of a previous investigation the strips were dried at atmospheric temperature for 15 min; this earlier experiment had shown that the percentage of organisms which survived the drying process fell rapidly during the first 10 min after spreading the film, but subsequently varied little with drying periods up to 1 hr.

Neutralizing agent. The neutralizing agent (2% lecithin and 3% Lubrol W) used by Garvie & Clarke (1955) was effective if allowed to act for 5 min (Whitt, 1958). The release of the solutions from the pipettes, and gentle rotation, provided sufficient agitation to mix the test solution and neutralizing agent. The neutralization of 20 ml of 200 p/m QAC solution was effected by 10 ml of the agent.

Enumeration of surviving organisms. When the squeegeeing technique used by Neave & Hoy (1947) and Garvie & Clarke (1955) proved ineffective on the modified

metal surface and in the absence of organic matter, a direct plating method was evolved to count surviving organisms. The treated strips were placed singly in Petri dishes and 10 ml of nutrient agar were poured over each. When cool the dishes were inverted and incubated at 37° for 24 hr. After cutting the agar around the strip the thin covering layer was peeled off leaving the bacterial colonies visible on the metal. Staining with carbol fuchsin for 30 sec improved the colour differentiation between the colonies and the metal surface. A cellophane graticule was laid over the contaminated area and the colonies counted using a dissecting microscope. The field of view (1 cm in diameter) was split into 10 vertical and 10 horizontal strips by the graticule. Since the organisms were evenly spread before drying it was found sufficiently accurate to count the colonies in only one vertical column (the maximum variation between columns was 3·5%) and multiply this number by 10 to give the count/field and then by the number of fields in the length of the contaminated area (usually 2·5–3·0). Only with counts below 1,500 colonies/strip did the uneven spread of survivors necessitate the counting of a complete field of view or even the whole strip.

Replication. Dilutions of the original bacterial suspensions used were plated in triplicate, while the mean of counts on 6 inoculated and dried strips gave the number of organisms still viable before treatment with the test solution (control counts); 6 replicated strips were also used to obtain the mean number of organisms surviving treatment.

Experiments and Results

In the main experiments the effects of time and temperature of exposure and of concentration of QAC were investigated. Times of treatment varied from 15 sec to 6 min according to the strength of QAC-detergent used. Since the manufacturer's instructions as to the temperature were not definite three temperatures were tried, 58°F (cold tap water), 100°F and 150°F. The concentrations tested were the full strength solution containing 200 p/m QAC, dilutions of which were made in tap water to give 150, 100 and 50 p/m.

Table 1. *The effect of temperature, concentration and time of exposure on the bactericidal activity of a QAC-detergent compound*

Expt.	Temp. (°F)	QAC concn. (p/m)	Percentages of survivors* after exposure for:							
			15 sec	30 sec	1 min	2 min	3 min	4 min	5 min	6 min
1	58	200	35·87	31·89	1·54	0·42	—	—	—	—
2	58	150	—†	64·50	33·73	2·49	1·76	0·56	—	—
3	58	100	—	66·32	58·17	54·69	16·58	3·20	—	—
4	58	50	—	—	—	60·89	47·11	31·58	28·84	28·88
5	100	200	49·92	18·52	6·81	0·04	0·04	—	—	—
6	100	150	—	46·30	23·41	2·33	2·33	0·55	—	—
7	100	100	—	—	55·90	20·41	4·09	3·58	1·58	—
8	100	50	—	—	—	46·47	30·84	21·71	20·93	16·81
9	150	200	6·64	5·79	0·37	0·18	0·03	—	—	—
10	150	150	—	7·59	2·58	0·27	0·08	0·01	—	—
11	150	100	—	—	30·23	3·59	3·23	0·12	0·05	—
12	150	50	—	—	—	11·32	14·87	13·69	11·91	2·86

* Means of 6 replicates. † —, not tested.

The results of the main experiments are presented in Table 1. In these, the conditions chosen were such as to cover the most important range of bacterial destruction. Exposure for 2 min to a stock solution of the QAC-detergent containing 200 p/m of QAC produced satisfactory results under all the conditions, the survivals being 0·42% at 58°F, 0·04% at 100°F and 0·18% at 150°F. There appears to have been little increase in effect with rise in temperature.

Lowering the concentration of the test solution from 200 to 150 p/m of QAC approximately doubled the time required to reach a similar level of survival at 58°F and 100°F. Only at 150°F was the drop in bactericidal efficiency due to the lower concentration compensated for by the rise in temperature. A 100 p/m QAC solution (half the recommended strength) showed a similar trend in behaviour and gave a 98–99% reduction in bacterial numbers only at 150°F and with a 5 min exposure period. The 50 p/m solution also acted more effectively as the temperature increased but even after 6 min exposure at 150°F the result was not satisfactory.

Table 2 gives the results obtained with a 200 p/m solution at the three temperatures and with 10% of milk added to the original bacterial suspension. After 2 min exposure

Table 2. *The effects of temperature and time of exposure on the bactericidal activity of a QAC-detergent compound in the presence of* 10% *added milk*

Expt.	Temp. (°F)	QAC concn. (p/m)	Percentages of survivors* after exposure for:				
			15 sec	30 sec	1 min	2 min	3 min
13	58	200	85·79	91·63	82·66	52·24	25·02
14	100	200	49·59	34·68	9·29	5·05	2·13
15	150	200	22·19	14·00	0·92	0·14	0·18

* Means of 6 replicates.

Table 3. *The coefficients of variation of the suspension counts and of the determinations of percentage survival on six replicate strips before and after exposure for various times to solutions of a QAC-detergent compound*

Expt.	Suspension count	Control counts*	15 sec	30 sec	1 min	2 min	3 min	4 min	5 min	6 min
1	6·61	8·39	53·76	41·22	131·40	77·62	—	—	—	—
2	4·74	7·82	31·82	98·54	96·70	59·31	89·21	—	—	—
3	3·19	34·74	55·33	129·40	51·89	64·76	106·00	—	—	—
4	8·18	9·16	—†	22·91	60·99	123·20	96·18	182·00	—	—
5	5·99	8·34	—	70·80	43·54	101·30	208·70	118·60	—	—
6	5·21	19·40	—	191·80	124·40	87·28	129·80	90·22	—	—
7	6·18	12·06	—	43·33	37·30	22·01	71·04	134·90	—	—
8	5·79	18·12	—	—	19·75	102·50	106·40	144·30	193·20	—
9	4·15	13·78	—	—	95·79	109·00	138·30	106·30	48·10	—
10	8·28	15·00	—	—	—	23·45	17·28	24·20	47·18	44·82
11	3·37	15·28	—	—	22·99	24·65	36·53	51·30	70·30	—
12	8·22	12·05	—	—	84·41	71·48	81·77	87·20	44·17	—
13	3·61	6·19	15·75	8·86	12·18	45·81	80·58	—	—	—
14	6·57	16·51	45·30	54·78	68·73	126·00	178·50	—	—	—
15	10·40	7·72	101·00	148·50	138·01	60·24	61·83	—	—	—

* Counts on strips before exposure. † Not tested.

the survivals were: 52·24% at 58°F, 5·05% at 100°F and 0·14% at 150°F. These results show that the rise in temperature caused a definite increase in the efficiency of the QAC detergent in the presence of 10% of milk. To effect a percentage reduction in numbers comparable with those obtained at any of the temperatures in the absence of added milk a substantial temperature rise would have been necessary.

Discussion

An idea of the reproducibility of the results is given in Table 3, which shows the coefficients of variation of the determinations of percentage survival presented in Tables 1 and 2 and of the suspension and control counts. The coefficients of variation of the counts of the original suspension were all small and relatively constant, and the control counts (i.e. the numbers of organisms surviving drying) were also satisfactory, except in one case (Expt. 3). But for the strips treated with QAC-detergent the coefficients range widely, from 8·9% to 208·7%. Because of this wide range of variation and the lack of replication of the complete experiments the values of percentage survival are considered to show only the trends of bactericidal action.

In their studies on the efficiencies of disinfectants applied to organisms on inanimate objects Stedman, Kravitz & Bell (1954) showed the erratic action of quaternary ammonium products. They lessened this effect by the use of replicate strips but irregular results still occurred. A similar state of affairs is apparent in the results in Tables 1 and 2, even though the figures given are the arithmetic means of the counts from six replicate strips. However, a reasonably accurate picture of the bacterial destruction would seem to have been obtained.

According to Clegg (1957) a minimum time of 2 min should be specified for chemical sterilization in the dairy industry. This period proved satisfactory with a stock solution of the QAC-detergent containing 200 p/m of QAC, irrespective of temperature, in the absence of organic matter. With 10% added milk present and a 2 min exposure time the solution required to be at 150°F to give the same extent of kill. At that temperature a solution containing 150 p/m of QAC gave a kill close to that given by the higher concentration, since the rise in temperature compensated for the drop in concentration. This compensation was not sufficient to enable the 100 and 50 p/m QAC solutions to produce satisfactory results, although the increases in bactericidal potency at the higher temperatures were considerable.

In these experiments the pH was always high and the water hardness low, and these conditions both conduce towards the efficiency of bactericidal action of QACs; but the limited agitation of the solutions applied to the infected strips and the nature of the modified metal surface itself would be expected to counteract those favourable conditions to some extent.

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THE FORMATION OF GENTIOBIOSE AND OTHER SACCHARIDES DURING PRESSURE-STEAM STERILIZATION OF A GLUCOSE-SALTS SOLUTION OF pH 6.8

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SUMMARY: Two samples of glucose of high quality were examined chromatographically for impurities in the form of saccharides other than glucose. One contained gentiobiose and at least three other sugars amounting to 35 mg/100 g of glucose; the other contained gentiobiose and at least four other sugars, the total quantity being 25 mg/100 g of glucose. Glucose from which these small quantities of impurities had been removed was employed, together with inorganic salts, to make up a medium suitable for the cultivation of moulds and the pH value was adjusted to 6·8. This medium was sterilized at 10 lb pressure for 15 min and it was afterwards found that a mixture of saccharides amounting to 125 mg/100 g of glucose had been formed, the principal constituent being gentiobiose; the remainder consisted of a monosaccharide and two oligosaccharides not yet identified. These sugars were not detected in similar glucose salts media which had not been heated but which had been sterilized by Seitz filtration.

Fischer (1890, 1895) showed that heating glucose with concentrated hydrochloric acid causes the formation of a mixture of substances including disaccharides. In recent years this so-called 'reversion' process has been increasingly studied under different experimental conditions. Pringsheim & Leibowitz (1924) and Pringsheim & Steingroever (1926) detected gentiobiose, Hurd & Edwards (1949) reported the formation of a trisaccharide and Myrback, Hammarstrand & Gelinder (1949) that of a tetrasaccharide, all as reversion products of glucose. Other information on this topic has been cited by Kent (1953) while Bacon & Bacon (1954) have made qualitative and quantitative observations on the products formed by heating glucose in N H_2SO_4, in N HCl, and also in the presence of oxalic acid. They confirmed the formation of isomaltose, first reported as a glucose reversion product by Berlin (1926), and they followed the course of oligosaccharide formation. They noted that acetic acid produced barely detectable effects on glucose. Quite recently, Sato, Watanabe & Aso (1958) have isolated laminaribiose from 'hydrol', which is the residual mother-liquor from the production of D-glucose by the acid hydrolysis of starch.

During recent studies in these laboratories of oligosaccharide formation by micro-organisms utilizing glucose as their source of energy, a supply of chromatographically pure glucose was required. Further, it was essential to be certain that other sugars were not formed by reversion of the glucose during the preparation and sterilization of the media. For these reasons the authors undertook experimental work to ascertain the behaviour of pure glucose when sterilized in the presence of mineral salts, in a medium of pH 6·8 prepared for the cultivation of mould fungi.

Experiments and Results

The purification of glucose

In order to ensure that the glucose used for this medium was chromatographically pure, two samples of high quality grades, namely B.P. 'Dextrosol' (Corn Products, Ltd.) and B.D.H. 'Analar' (British Drug Houses, Ltd.), were examined. Paper chromatograms prepared from samples of each grade failed to show the presence of any sugar other than glucose. Since the failure to detect any other sugar may have been caused by the masking action of excess glucose, treatment was then applied to two portions of these grades, each of 300 g, with the object of fractionating out any other saccharide or saccharides which might be present in very small quantity. The two 300 g quantities were dissolved separately in distilled water to form solutions of 20% (w/v) concentration, which were then subjected to charcoal-'Celite' chromatography essentially as described by Bacon (1954). For elution, water and a mixture of water (75 vols.) with ethanol (25 vols.), respectively, were employed. The water eluates were each concentrated under vacuum below 35° until the quantity of carbohydrate in solution was approximately 10% (w/v). The aqueous ethanol eluates were concentrated under similar conditions, and the residues were freeze dried, weighed, and subjected to paper chromatography. From the 300 g of Dextrosol there was thus separated 410 mg of material consisting partly of inorganic matter from the 'Celite' (303 mg) and partly of a mixture of sugars (107 mg) of which at least three were saccharides higher than monosaccharide. The 300 g of Analar glucose similarly yielded 378 mg of material consisting of inorganic substances (303 mg) together with saccharides (75 mg), of which there were five, at least four being oligosaccharides. Two of the latter had very low R_G values and were present merely in traces. Table 1 shows the R_G values of these substances when the solvent was *n*-propanol-water-ethyl acetate (7:2:1 v/v, Gross & Albon, 1953).

Table 1. *The R_G values of saccharide impurities detected by paper chromatography with 7:2:1 (v/v) n-propanol-water-ethyl acetate in material separated from two high quality samples of glucose*

Sample of glucose	R_G values of saccharides				
Dextrosol B.P.	0·15*	—	0·49†	0·62	0·78*
'Analar' glucose	0·15*	0·33*	0·49†	0·62	0·78*

* Detected only in traces. † Using the same solvent the R_G value of authentic gentiobiose was 0·48.

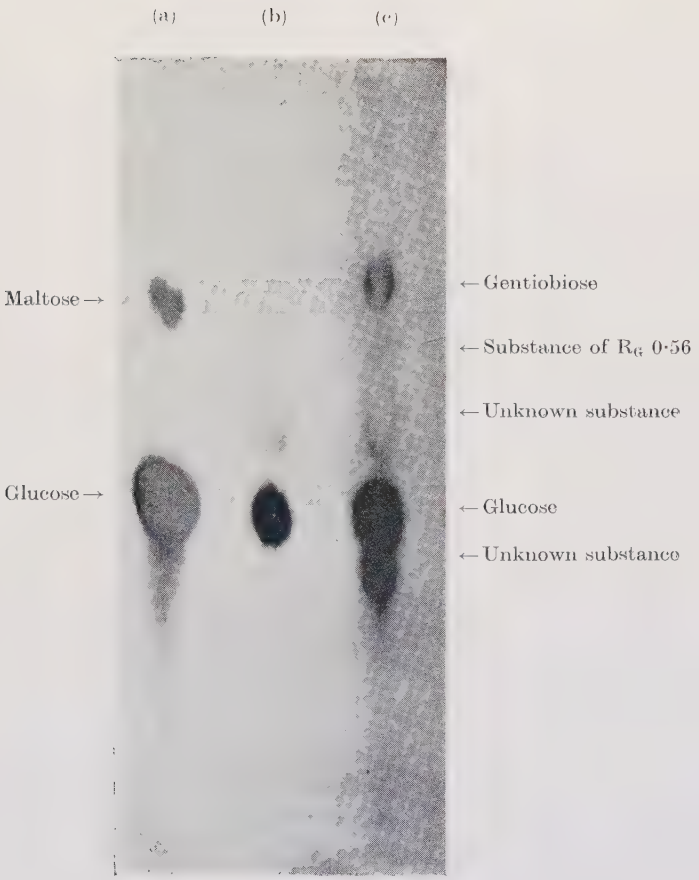
The saccharides having R_G values of 0·49 and 0·62, present in the disaccharide region, were separated by band chromatography on 3 MM Whatman paper, using the *n*-propanol-water-ethyl acetate solvent. Authentic gentiobiose had an R_G value of 0·48 in this solvent. The benzylamine derivative of the substance of R_G value 0·49 was prepared by the method of Bayly & Bourne (1953) and was indistinguishable from the benzylamine derivative of authentic gentiobiose in chromatographic preparations. When the material whose R_G value was 0·49 was completely hydrolysed by treatment with 2N H_2SO_4 for 2 hr at 80°, the hydrolysate gave only one spot detectable by paper chromatography: this had an R_G value corresponding to that of

glucose. This evidence indicates that the substance of R_G value 0·49 was gentiobiose. The second disaccharide, of R_G value 0·62, was also completely hydrolysed and gave only one spot, whose value corresponded to that of glucose. The mobility of this diglucosaccharide lay between those of sophorose (R_G value 0·65) and maltose (R_G value 0·59); there was insufficient for further identification tests.

After two lots of glucose obtained from the water eluates from the charcoal-'Celite' columns used in the process of purification described above had again been submitted to the purification process and passed a second time through charcoal-'Celite', chromatographic examination showed that in each case the saccharides which previously had been detected as impurities were now absent, though there was a trace of the sugar of R_G value 0·78, which gave a very weak spot in the monose region close to glucose. This monosaccharide was present in the glucose after its first passage through charcoal-'Celite'.

Reversion products of glucose, formed in the presence of mineral salts during heating at 10 *lb steam pressure*

A quantity (300 g) of this highly purified glucose was now employed to make up a medium of the following composition (g/l): NH_4NO_3, 3·00; $NaH_2PO_4.2H_2O$, 7·30; K_2SO_4, 0·22; $MgSO_4.7H_2O$, 0·50; $ZnSO_4.7H_2O$, 0·05; $FeCl_3.6H_2O$, 0·16; glucose, 170. The constituents were dissolved in distilled water and the pH value adjusted with NaOH to 6·8. This medium was then divided into three equal portions. One was submitted to chromatographic treatment with charcoal-'Celite' and the eluates concentrated under low pressure and examined as before by paper chromatography. Only glucose and a trace of the monose of R_G value 0·78 were detected. The dried aqueous ethanol eluate, which consisted almost entirely of inorganic matter, weighed 99 mg. Another portion was Seitz filtered and then treated in the same way. Again, only glucose and a trace of the monose of R_G value 0·78 were detected, and the dried inorganic residue from the aqueous ethanol eluate weighed 101 mg. The third portion was sterilized in steam at 10 lb pressure for 15 min, cooled and then submitted to chromatographic treatment with charcoal-'Celite' as in the other cases. When the aqueous ethanol eluate was chromatographed on paper it yielded four spots in addition to that given by glucose. The main spot corresponded to gentiobiose. In the disaccharide zone was one other spot, of R_G value 0·56. One of the two remaining spots had an R_G value of 0·78: this substance had been detected previously in the purified glucose. The other had an R_G value greater than that of glucose (Plate 1). The identification of the substance giving the main spot as gentiobiose (R_G value 0·48 when in the *n*-propanol-water-ethyl acetate solvent) was confirmed by the chromatographic procedure of Jermyn & Isherwood (1949), in which the solvent is pyridine-ethyl acetate-water (1:2:2 v/v). The benzylamine derivative of the substance was also compared chromatographically with the benzylamine derivative of authentic gentiobiose. In both cases the correspondence was exact. The dried residue from the aqueous ethanol eluate weighed 226 mg, of which 101 mg represented inorganic substances derived from the 'Celite', showing that during the sterilization of 100 g of pure glucose in the mineral salts medium there was formed 125 mg of a mixture of saccharides consisting mainly of gentiobiose with lesser quantities of unidentified oligosaccharides.



KHAN, A. W. & WALKER, T. K. — THE FORMATION OF GENTIOBIOSE AND OTHER SACCHARIDES DURING PRESSURE-STEAM STERILIZATION OF A GLUCOSE-SALTS SOLUTION OF pH 6·8. PLATE 1

Discussion

Although it has long been known that reversion of glucose occurs when it is heated with acids, so far as the authors are aware definite information on oligosaccharide formation during the sterilization of simple glucose media, such as are used for mould fungi, is lacking. From the results now presented it is clear that even a short period of sterilization at 10 lb pressure in the presence of simple inorganic salts at pH 6·8 is sufficient to ensure the formation from glucose of a number of saccharides, in quantity up to a total of 125 mg/100 g of glucose. On the other hand the formation of these sugars is entirely avoided when the medium is sterilized in the cold by passage through a Seitz filter. The production of oligosaccharides during the steam sterilization of glucose-containing media must therefore be borne in mind when experiments concerning the formation of oligosaccharides from glucose by living micro-organisms or enzyme systems are being arranged.

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Explanation of Plate

Comparison chromatograms of saccharides in 7:2:1 (v/v) *n*-propanol-water-ethyl acetate.

(a) Authentic specimens of maltose and glucose.

(b) 'Analar' glucose after purification by charcoal-celite and sterilization by Seitz filtration, when in a salts solution.

(c) 'Analar' glucose after purification by charcoal-celite and sterilization at 10 lb for 15 min in a salts solution.

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VACUUM DRYING OF STEAM STERILIZED DRESSINGS

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SUMMARY: The degree of vacuum and time of application needed to dry steam sterilized dressings has been investigated. If the steam supply is dry, and the pressure in the drying stage is reduced to an absolute pressure of 50 mm Hg, dressings will be cool and dry without holding the vacuum, provided they are not damp before sterilization.

Sterilization of dressings in steam sterilizers is usually carried out in four stages: first, removal of air; second, exposure to steam under pressure; third, drying and fourth, admission of sterile air. Recently, papers have appeared dealing with the first two stages (Alder & Gillespie, 1957; Knox & Penikett, 1958) and attention has been drawn to faults occurring in the fourth stage (Howie & Timbury, 1956). The drying stage does not seem to have received the same attention. This is surprising because complaints of wet operating-theatre dressings and gowns are often heard. Perkins (1956) devotes a short section of his book to the subject and suggests that attention should be paid to the quality of the steam.

This paper is concerned with the drying of goods after steam sterilization in what is usually called a dressing sterilizer. These machines may be used for mixed loads which include instruments and bowls, etc., as well as dressings. The term dressings is intended to include not only surgical dressings but other items made of cotton material such as towels, gowns and masks.

There are several methods which are alleged to dry dressings after steam sterilization. The dressings can be allowed to remain in the sterilizer with the door 'cracked' open, to be dried by heat from the jacket, or air can be drawn through the chamber by a pump so that water vapour is removed by being entrained with the air. In another common method the pressure in the chamber is reduced below atmospheric pressure and held there for periods varying from 15 min to 1 hr or longer. The first two methods tend to dry dressings on the outside but leave the inside damp. The use of the 'cracked door' must be condemned because of the risk of contaminating the load with unsterile air. The third method depends on the degree of vacuum obtained. Thus if a vacuum of 10 in. Hg is drawn then only one-third of the free steam in the chamber will be removed. However long the load is left in the chamber two-thirds of the steam will remain.

Most modern sterilizers employ the third method and we set out to establish the degree of vacuum and the time of application needed to dry sterilized dressings. In order to do this we had to relate absolute and subjective dryness so that we had some

measurable standard for reference. Once what is meant by 'dry' had been established in physical units the problem of the degree and duration of vacuum necessary to obtain dryness could be approached theoretically.

Henry (1959) has discussed the theory of drying after exposure to steam under pressure. He predicted that dressings would return to substantially the same state after sterilization as they were before. The extent to which this is true will depend on how reversible the system is. A completely reversible system would be one in which no heat entered or left the dressings other than through the medium of the steam. That is, they are heated by the latent heat of condensation and they are cooled by giving up the latent heat of vaporization. For this theory to apply in practice all the air must be removed from the load and chamber before the admission of steam, the steam must be exactly at the phase boundary, and after sterilization all the steam must be removed and replaced by air in the same state as that removed at the beginning of the process. Clearly, these ideal conditions cannot be met in practice. It would be uneconomical to provide pumps which could remove all the air and all the steam. Furthermore, for a long time to come hospitals are likely to use dressing drums which impede the removal of air and interfere with the drying process by allowing condensate to collect in them. In our experiments we have attempted to find the degree of vacuum required to dry dressings when they have been packed in drums standing on their bases, although better results would have been obtained with drums correctly positioned on their sides and still better results if no drums had been used at all. This was done deliberately so as to set up the worst conditions likely to be found in practice. We have already established that predictable and rapid sterilization can be obtained if the absolute pressure in the chamber before sterilization is reduced below 20 mm Hg (Knox & Penikett, 1958). The pressure was reduced to this level in all our experiments and the results will only apply if these conditions of pre-vacuum are used.

Materials and Methods

The sterilizer. The sterilizer was a cylindrical horizontal jacketed chamber of approximately 11 ft^3 capacity. The chamber and the jacket were independently fed with steam at 20 lb/in^2. The steam had passed through two separators in series before reaching the chamber. A 2 in. pipe connected the chamber through a valve to a water cooled condenser in series with an oil sealed rotary pump. For a fuller description of the apparatus see Knox & Penikett (1958). The jacket was left filled with steam during the drying stage but we have since found that this had little or no influence on drying.

The load. Experiments were carried out with one or a number of drums in the chamber. A drum, which we call the standard drum, was present in each experiment. It was a cylindrical, polished, stainless steel dressing drum, of diameter 11 in. and height $8\frac{1}{2}$ in., with eight $\frac{1}{2}$ in. holes equally spaced around the side wall. The drum was lined with two thicknesses of huckaback towelling and loaded with eight 20×30 in. huckaback hand towels folded so that each presented 12 thicknesses.

In the course of preliminary experiments the best results were obtained with the standard drum placed in the lower front position, with nothing else in the sterilizer.

When the standard drum was placed in the lower back position with five other drums in the chamber the worst results were obtained. Included in the centre of the standard drum in each experiment was a sample of towelling 4 in. square, which was used for moisture determination. In every case the drums were stood on their bases. The warp of the huckaback towelling was American cotton and the weft Egyptian cotton, both Lancashire spun condenser yarn.

Steam dryness measurement. A $\frac{1}{4}$ in. diameter copper tube was fixed vertically so as to project 12 in. into the chamber from the top. Twelve $\frac{3}{64}$ in. diameter holes were drilled in the wall of the tube on the side facing the rear of the sterilizer, whence the steam entered the chamber. The tube led to the outside via a valve and a well-lagged extension from which steam could be sampled.

After the steam pressure in the sterilizer had reached a steady level the valve on the sampling tube was slightly opened. A 1 pint Dewar flask was two-thirds filled with a known quantity of cold water at a measured temperature. The sampling tube was then introduced into the flask and steam allowed to blow rapidly into the water, care being taken that no steam was lost out of the flask. When the temperature of the water reached about 170°F the flask was removed and the weight and temperature of the water were again determined. The mass of steam condensed was calculated from the increase in weight of water. The heat which it contained was found from the rise in temperature. Thus the latent heat of the steam in B.t.u./lb could be calculated and compared with that of the value for dry saturated steam quoted in Callendar's Steam Tables (1939). The ratio of one to the other is the dryness fraction.

Moisture determination. The moisture content of dressings was determined in two ways. In the first, the moisture content of the 4 in. square sample of towelling was determined by measuring its vapour pressure under vacuum, and referring to a curve relating equilibrium vapour pressure to percentage moisture content previously obtained by an absolute method (Fig. 1). A modified Edwards moisture determination apparatus MDA/2 (Fig. 2) was used. The procedure was as follows. As soon as the sterilizer door was opened a sample of towelling was removed and placed in the specimen tube. The tube and the trap were surrounded with a mixture of solid CO_2 and methylated spirit. The pressure in the apparatus was reduced to 0·02 mm Hg, the tap between the manometer arms (A in Fig. 2) closed and the refrigerant mixtures surrounding the tube and trap were removd. After 20 min the vapour pressure was read as the difference between the oil levels in the manometer arms. This figure, corrected for temperature and converted into mm Hg, was entered in the predetermined curve (Fig. 1) and the moisture content read off. This method was convenient and accurate but could not cope with moisture contents over 10%. In experiments in which we wished to measure high moisture contents a weighing method was used.

In the second method, the weighing method, used to test Henry's reversibility theory and to check on the first method, dressings of a known dry weight were packed in a weighed drum, allowed to take up moisture, and the drum and its contents weighed. The percentage moisture content was calculated from the increase in weight of the dressings. This method appears more simple than the first but suffers from certain disadvantages. When dressings are packed in a drum and sterilized the moisture content at the end of the process is not uniformly distributed throughout

the contents of the drum; therefore the weighing method gives only an average value. Sometimes, due to condensation, the outer layers may be 2 or 3% wetter than the centre. This was established by placing samples in different parts of the drum and measuring their moisture contents by the vapour pressure method. Further errors are likely, due to buoyancy effects and losses due to evaporation during weighing (Stephenson, 1938).

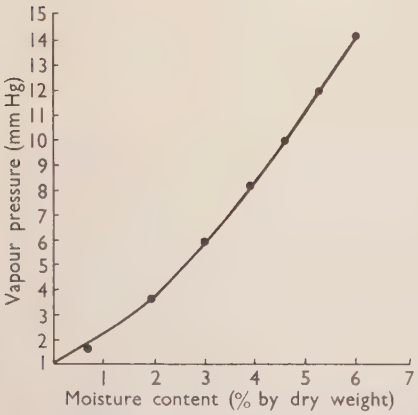

Fig. 1. The relation between the equilibrium vapour pressure of dressings and their moisture content at 25°.

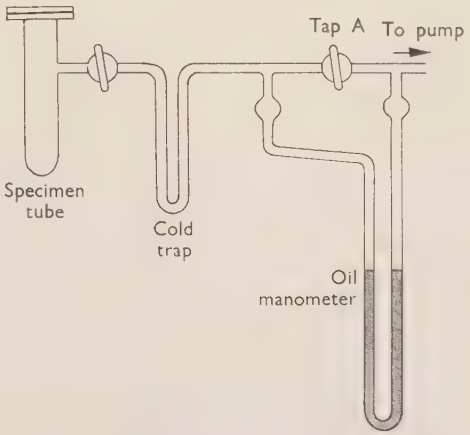

Fig. 2. The modified Edwards moisture determination apparatus MDA/2.

The influence of the degree of vacuum on the drying process. The pressure in the chamber was reduced to 20 mm Hg before steam was admitted. The steam was applied at 20 lb/in.^{2} for 10 min. The steam was then turned off and the pressure in the chamber reduced to the required level. As soon as this was reached air was admitted to the chamber, and the door was opened when atmospheric pressure was reached. The sample of towelling was removed from the standard drum and placed immediately in the moisture determination apparatus. The level of the vacuum reached in the drying process was varied from atmospheric pressure to 20 mm Hg abs. The experiments were conducted with the standard drum alone in the chamber in the lower front position and also with the standard drum in the lower back position with five other drums in the chamber, that is, the positions and loads which gave respectively the best and worst results. The moisture contents of the samples of towelling were not measured before sterilization in each case but the samples were kept in an atmosphere of approximately 45% r.h. which means (see below) that they had a moisture content of about 5%.

The reversibility of the steam sterilizing process. In order to see how far Henry's theory held in our imperfect system the chamber was loaded with a drum full of dressings and sterilized with steam at a pressure of 20 lb/in.^{2} for 10 min after an initial chamber pressure of 20 mm Hg had been obtained. The steam was pumped out at the end of the process until the pressure in the chamber was again 20 mm Hg abs. The initial moisture content of the dressings was varied from nearly dry to

saturated. Cotton becomes saturated when it contains about 20% by dry weight of water. The moisture determinations for these experiments were carried out by the weighing method.

The moisture content of dressings feeling 'dry'. There is no need to dry towels to a lower moisture content than they would acquire by exposure to the atmosphere during storage. The r.h. of our store rooms was about 45%. Towels kept in such an atmosphere appear crisp and are dry to touch, whereas those kept in an atmosphere of high humidity are limp in appearance and feel damp. Atmospheric humidity does not appear to influence the subjective assessment of a towel's dryness, except that wet towels in a dry atmosphere feel colder than those in equilibrium with the atmosphere.

To relate absolute and subjective dryness, towels were first allowed to come to equilibrium with atmospheres of known relative humidity by keeping them in desiccators with sulphuric acid of predetermined concentrations. Several observers were then asked to say whether a towel was wet or dry. Moisture determinations were also carried out on the towels by the vapour pressure method. Thus subjective dryness could be related to moisture content and equilibrium relative humidity.

Results

Steam dryness fraction. The steam in the chamber was sampled on 27 occasions and the dryness fraction was calculated. The mean value was 0·92 (standard deviation, 0·04).

The degree and duration of vacuum necessary in the drying process

The results of the two sets of experiments are displayed graphically. Fig. 3a is a graph of percentage moisture content of the towel samples taken from the standard drum against the absolute pressure in the chamber at the end of the drying stage, the standard drum having been alone in the chamber in the lower front position. For example, when the pressure in the chamber, after the steam had been turned off, was reduced to 510 mm Hg abs. (approximately 10 in. of vacuum), the sample contained about 7% of its dry weight of water. When the pressure was reduced to 20 mm Hg (approximately 29 in. of vacuum) the moisture content was only 2%. Once the vacuum pump had reduced the pressure in the chamber to a particular figure it was not held. Thus the duration of the drying stage was only that required for the pump to reduce the pressure to the required level. In the case of the high degrees of vacuum such as 10 mm Hg the time taken was between 2 and 3 min. For lesser degrees of vacuum the time was proportionately shorter.

Fig 3b shows the results obtained when the standard drum was in the lower back position in company with five other drums in the chamber. The results show that the towel samples were wetter when the chamber was fully loaded and the standard drum was in the rear. The time taken for the pump to reduce the pressure in the chamber to 10 mm Hg was nearly 5 min. If it is assumed that a sample is 'dry' when its moisture content is below 5% (see below), then in the case of the single drum the load was dry when the pressure in the chamber had been reduced to about 300 mm

Hg (18 in. of vacuum). In the case of the full chamber the same degree of dryness was reached when the pressure was reduced to 200 mm Hg (21 in. of vacuum).

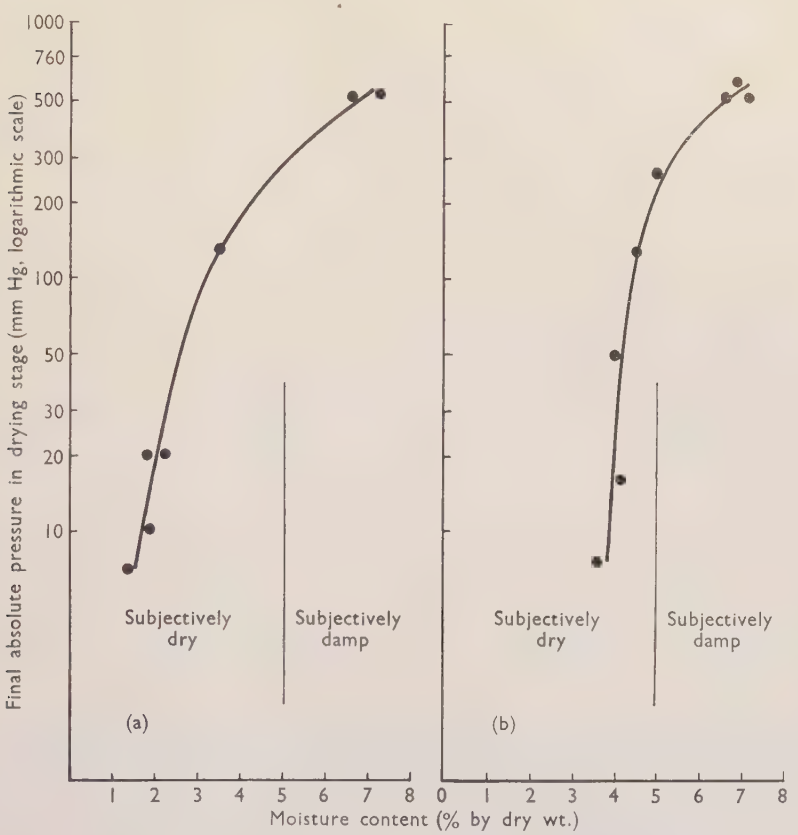

Fig. 3. The moisture content of towel samples after different degrees of vacuum in the drying stage: (a) with only the standard drum in the chamber; (b) with the standard drum plus five other drums in the chamber.

The reversibility of the steam sterilizing process

Fig. 4 is a graph which shows the percentage moisture content of dressings packed in a drum before and after sterilization for 10 min with steam at a pressure of 20 lb/in^2. The moisture content was calculated by the weighing method. The broken line represents the curve which would be obtained if there were perfect agreement with Henry's theory. The initial moisture content varied from 0–19%. The greatest change in the amount of water present in the dressings was 1·5%. In several cases the changes were only a fraction of 1%. If dressings and gowns, etc. are properly dried after laundering they will have a moisture content between 2 and 5%. In 6 experiments in which the initial moisture content was in this range, the final reading after steaming returned to within 1%.

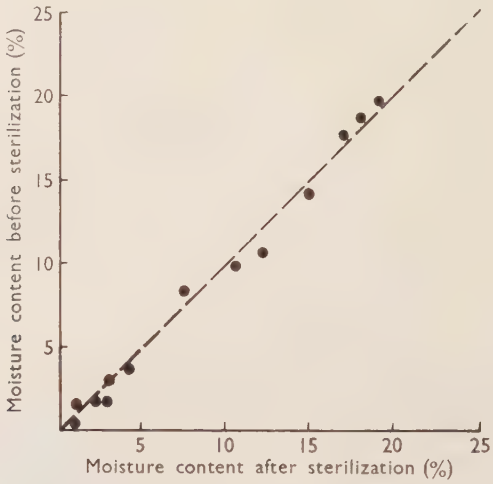

Fig. 4. The relationship between the percentage moisture contents of dressings before and after sterilization. The broken line represents the relationship predicted by Henry.

Dryness

The subjective assessments of dryness of towels of known moisture content and equilibrium r.h. are shown in Table 1. Three observers made an assessment of each different condition and agreed on their description. From these results it can be said that a cotton towel which feels dry to the touch contains less than 5% of its dry weight of water.

Table 1. *The relationship between absolute and subjective measures of the dryness of towels*

Condition of towel according to		Condition by 'feel'
Equilibrium r.h. (%)	Moisture content (% of dry wt.)	
44	4·8	Dry
47	5·0	Dry
60	6·0	Very slightly damp
70	7·0	Slightly damp
80	8·4	Damp

Discussion

When cotton from which air has been removed is exposed to steam its moisture content will rise rapidly to a point of temporary equilibrium. The function of steam is to heat the cotton and its water content by condensing. Thus a greater quantity of steam will be needed to condense and give up its heat to raise the temperature of wet material than dry. It has been calculated (Henry, 1959) that if the initial moisture content is zero then the equilibrium moisture content during steaming will be 6%. If the material initially contains 5% moisture then the equilibrium moisture content

will be 12%. This is the amount of water that dressings would be expected to contain if they could be removed instantaneously from the steam. In two experiments in which the initial moisture contents were 4·3 and 4·5% they contained 10·5 and 11·4% respectively after steaming, the dressing having been removed as quickly as the steam could be turned off and the door opened. Allowing for the fact that some evaporation would have taken place while the steam pressure was falling to atmospheric pressure these figures agree fairly well with the theoretical calculations. If the process is allowed to complete the cycle of high initial vacuum, steam under pressure followed by high vacuum drying, then the graph in Fig. 4 shows, as Henry predicted, that the moisture content of the dressings will return to near their original value; that is, water is lost as they cool by evaporation just as it was gained as they were heated by condensation. Not only will the moisture content return to its original value but so also will the temperature.

In centrally heated hospitals, dressings will be placed and stored ready for sterilization in atmospheres of about 45% r.h. Fabrics differ in the amount of moisture they contain in equilibrium with this humidity. Most dressings, towels and gowns are made of cotton fabrics which will contain about 5% of water in equilibrium with this humidity. The graph in Fig. 5 shows the predicted moisture regain of soda-boiled cotton for different conditions of r.h. The results represented by the circles plotted on the graph were obtained in experiments on towel samples. They fall near the predicted curve.

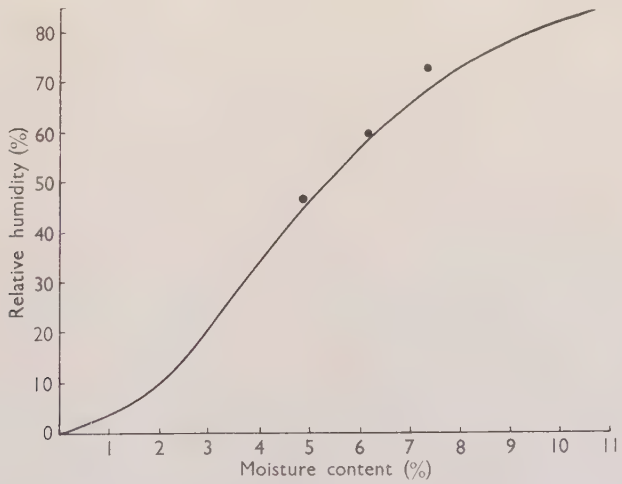

Fig. 5. The moisture regain of soda-boiled cotton for different conditions of relative humidity at 25°. The curve was drawn from a nomogram constructed by Henry. The three full circles represent results obtained experimentally with the huckaback towelling described in this paper.

In the experiments in which the sterilizer was loaded with 6 drums the moisture content was determined after allowing the pressure in the drying stage to fall to different levels. Fig. 3 shows that only a moderate vacuum was necessary to dry the dressings to less than 5%. There is an advantage in reducing the pressure still further

because bowls and instruments, if sterilized together with the dressings, will be cooler. Furthermore, if drums are used the small quantity of steam remaining in them will condense and wet the outer layer of the contents when the drum and its contents cool. This does not occur if the absolute pressure is reduced to 50 mm Hg or below.

It is emphasized that these results were obtained without holding the vacuum. The time for the drying stage need only be that required to reach 50 mm Hg abs., which in our experiments varied with the load from 2 to 4 min. Our results were obtained with dry steam and could not have been obtained with wet steam. In this connection it must be understood that dressings which are initially wet (e.g. because of wet steam) cannot be dried, except on the outside, by prolonged exposure to vacuum alone; they must be supplied with heat of vaporization. This heat can be supplied by a jacket; but if the dressings are dry initially then clearly no heat from any source is required.

Although many hospital sterilizers are not fitted with equipment which can draw a high vacuum, most can draw a vacuum which would be expected from our experiments to produce dry, but not cool, dressings in a few minutes. Since this result is often not obtained, it must be suspected that the steam supply is wet. This is simply rectified by the addition of separators close to the chamber.

The results described in this paper were obtained during the course of work carried out at the request of the Medical Research Council Working Party on Steam-Pressure Sterilization.

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THE MYCOLOGICAL FORMATION OF FAT IN CULTURES GROWN WITH AGITATION AND AERATION

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SUMMARY: A description is given of a stainless steel fermentation vessel of Waldhof type which was used for the cultivation of moulds for the purpose of obtaining fat. In this vessel *Aspergillus nidulans, Penicillium javanicum* and *Pencillium spinulosum* were grown in defined media containing inorganic salts and glucose, with vigorous agitation and aeration. In a medium containing glucose (90 g/l.) *A. nidulans* produced 4·4 g of fat/l. and the fat coefficient (g of fat formed/100 g of sugar utilized) was 8·9; while *P. javanicum* in this medium produced 3·6 g of fat/l. and the fat coefficient was 7·6. In a different medium containing glucose (100 g/l.) *P. spinulosum* produced 11·4 g of fat/l. and the fat coefficient was 12·0.

The authors have been interested for a number of years in the production of fat by moulds and have established conditions favourable to the synthesis of fat by *Aspergillus nidulans*, *Penicillium javanicum* and *Penicillium spinulosum* growing in surface culture (Garrido & Walker, 1953; Garrido, Woodbine & Walker, 1953; Gad & Walker, 1954; Garrido & Walker, 1956). On the basis of these data, acquired from the study of static cultures, experiments were devised to ascertain the capacities of these three moulds to accumulate fat during cultivation in agitated media. In what little work has been published hitherto on the topic of fat production by moulds grown in deep culture, the emphasis seems to have been on the possibilities of using wastes of low carbohydrate content as substrates for species of *Fusarium* and of *Mucor*. References to the experiments of several workers on these lines were given recently by Garrido, Gad & Walker (1958) when reporting their own results with *A. nidulans*, *P. javanicum* and *P. spinulosum*, cultivated with shaking in defined media containing glucose or sucrose as the source of carbon. These results were sufficiently promising to warrant extension of the experiments and further work has now been carried out on a larger scale. Since the ultimate object of these enquiries is to determine whether fat synthesis by moulds utilizing cheap forms of carbohydrate can be placed upon an economic basis, it was necessary to devise a small pilot plant for the purpose and experimental studies with a stainless steel fermentor of Waldhof type are now described.

Experimental Methods

Description of the stainless steel fermentor

The design of this fermentor was based on that of a fermentation vessel described by Brown & Peterson (1950). The vessel, which was of 75 l. capacity, was made for us by F. Pedley, Ltd., Middleton, Lancashire. It is shown in Plate 1 set up in conjunction with a vacuum pump and compressor of type IV (Edwards High Vacuum

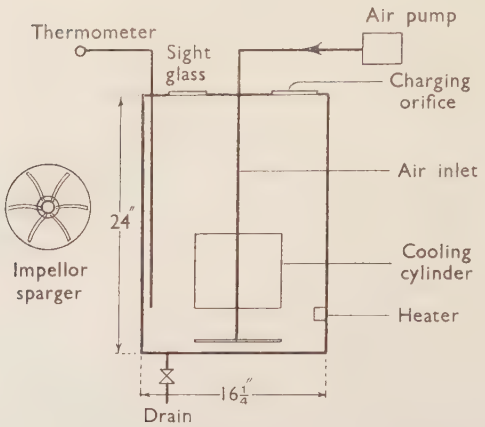

Fig 1. Diagrammatic representation of the fermentor.

Ltd., Crawley, Sussex), which can deliver 70 l. of air/min, together with a 0·75 h.p. DC motor, turning at 1440 rev/min, and a thermostat; all of which are assembled on a steel platform. The motor drives the impellor shaft by means of a V-belt at 2:1 reduction. The central interior cylinder shown in the line diagram (Fig. 1) was made with a jacket through which could be circulated a stream of water, should cooling be necessary.

Method of using the fermentor

The fermentor was washed with hot sterile water and then with a mixture of equal volumes of ethanol and water, after which steam was blown through the vessel for 5 min. The vessel was cooled under positive sterile air pressure and was immediately charged under aseptic conditions with 18 l. of sterile medium. The addition of 2 l. of inoculum was made immediately, agitation by the impellor at 720 rev/min was started, and aeration down the shaft of the impellor at 0·5 l. of air/l. of medium/min was commenced.

The air which was forced into the vessel was first sterilized by passage through a stainless steel cylinder (internal dimensions 10×4 in.) containing cotton wool. This had been sterilized at 20 lb steam pressure for 30 min. After leaving the filter the air was led through a saturator containing sterile water (at a temperature between 35° and 50° according to the ambient temperature and also to the temperature inside the fermentor) and finally down the shaft of the impellor.

When working with *A. nidulans* the medium in the fermentor was held at 30°, but when *P. javanicum* or *P. spinulosum* was employed the temperature initially was 25° and usually rose to 26–27°, at which it was then maintained.

At the commencement of vigorous growth in the fermentor foaming was not pronounced, and since Brown & Peterson (1950) indicated that foaming helps to provide good aerobic conditions the surface-active agent Comprox A (Shell Chemicals, Ltd.) was added to the extent of 0·5 ml/l. of medium. This gave rise to a large amount of foam and to control this oleic acid, 0·1 ml/l. of medium, was added at the commencement of incubation. When strong growth had developed, further foaming did

not occur. Although the volume of the fermentor was 75 l., usually only 20 l. of medium were used in each experiment, for it was found that when a charge of 30 l. was employed the foaming was difficult to control. Samples withdrawn for analysis during the course of the experiments were each of 250 ml. The treatment of these samples and the subsequent estimations of sugar, dry felt and fat were carried out exactly as described by Garrido, Gad & Walker (1958) when reporting their work with shaken cultures of these moulds.

Particulars of the organisms

The strains of *Aspergillus nidulans* Eidam, *Penicillium javanicum* v. Beyma and *Penicillium spinulosum* were those which had been used in previous work (Woodbine, Gregory & Walker, 1951) and were maintained in a similar manner.

Culture media and methods of cultivation

The defined media found suitable for the growth of these moulds in shaken culture have been described by Garrido, Gad & Walker (1958) and were adopted in the present investigation. Thus, for all three moulds the basal medium contained (g/l.): $NaH_2PO_4.2H_2O$, 0·180; $MgSO_4.7H_2O$, 0·060; K_2SO_4, 0·055; $ZnSO_4.7H_2O$, 0.013; $FeCl_3.6H_2O$, 0·040. To this there was added a source of nitrogen, which in the case of *A. nidulans* was $NaNO_3$ at 2·560 g/l., while for *P. javanicum* and *P. spinulosum* it was NH_4Cl at 1·600 g/l. These two media were completed by additions of glucose at 90 g/l. for *A. nidulans* and *P. javanicum*, and at 100 g/l. for *P. spinusolum*, and the media were then employed for the experiments recorded in Tables 1, 2 and 3.

Modified forms of the basal medium were later tested in further experiments with *P. spinulosum* and gave the results shown in Tables 4 and 5.

Preparation of the inoculum

For each charge of medium in the steel fermentor the inoculum was prepared as follows. Four 2 l. conical flasks with 5 cm diameter necks were each charged with 50 ml of malt wort agar. After sterilization, and when the agar medium had cooled to 45°, 2 ml of a suspension of spores of the mould were added to each flask and the flasks were then rotated while the agar was setting so as to give a thin layer over a large area. After incubation for 4 days at 27° (30° in the case of *A. nidulans*) the flasks were left at room temperature for a few days until sporulation was well advanced. To each flask there were then added 500 ml of the same sterile medium as was to be used in the fermentor, and the flasks were placed on a shaking machine in an incubator room. The machine was of the rotating type and operated at 66 rev/min with a 3 in. throw. After incubation in these conditions for 24–36 hr the cultures were usually ready for addition to the sterile medium in the fermentor. As the volume of this charge was normally 18 l., with the 2 l. of inoculum the total charge became 20 l.

Results

In shaken flask cultures of *A. nidulans* prepared with the same medium as used in the present work, Garrido, Gad & Walker (1958) obtained 8·9 g of fat/l. and the fat coefficient (g fat/100 g glucose utilized) was 14·0, while in the same basal medium and under the same conditions *P. javanicum* afforded 3·7 g of fat/l. and the fat coefficient was 7·9. In the present work the best result with *A. nidulans* (Table 1) was

Table 1. *Details of the growth of* A. nidulans *in agitated and aerated culture in a basal salts solution plus* $NaNO_3$ (2·560 *g/l.*) *and glucose* (90 *g/l.*)

Period of incubation (hr)	Glucose utilized (g/100 ml)	Mycelial felt (g/100 ml)	Fat produced		
			(g/100 ml)	(g/100 g of dry felt)	(g/100 g of glucose utilized)
24	2·4	0·70	0·09	12·8	3·7
48	3·2	0·98	0·17	17·4	5·3
60	4·3	1·10	0·29	26·4	6·7
72	4·5	1·45	0·39	26·8	8·6
96	4·9	1·58	0·44	27·0	8·9

Table 2. *Details of the growth of* P. javanicum *in agitated and aerated culture in a basal salts solution plus* NH_4Cl (1·600 *g/l.*) *and glucose* (90 *g/l.*)

Period of incubation (hr)	Glucose utilized (g/100 ml)	Mycelial felt (g/100 ml)	Fat produced		
			(g/100 ml)	(g/100 g of dry felt)	(g/100 g of glucose utilized)
48	1·2	0·32	0·04	12·5	3·3
64	2·4	0·51	0·14	27·4	5·8
91	3·2	0·74	0·29	39·2	9·0
115	4·5	0·93	0·34	36·5	7·5
151	4·7	0·95	0·36	37·9	7·6

4·4 g of fat/l. with a fat coefficient of 8·9, while the highest yield with *P. javanicum* (Table 2) was 3·6 g of fat/l., the fat coefficient being 7·6. According to these figures the efficiency of *P. javanicum* as a fat-forming agent was of the same order in the fermentor as in the shaken flasks, but the fat-forming capacity of *A. nidulans* when in the fermentor was very much less than it had been in shaken cultures. With *A. nidulans* there was, moreover, a tendency for the new mycelial growth to adhere to the wall of the fermentor and particularly to the inner cylinder, with the production of dense sticky aggregates. This form of growth probably received insufficient oxygen, with consequent diminution in the yield of fat.

P. spinulosum in the fermentor, in the basal medium that was used for the two other moulds, gave a yield (6·0 g of fat/l. with a fat coefficient of 12·8, Table 3) very

similar to that (6·3 g of fat/l., fat coefficient 10·3) which it had afforded when grown in this medium in shaken culture.

Table 3. *Details of the growth of* P. spinulosum *in agitated and aerated culture in a basal salts solution plus* NH_4Cl (1·600 *g/l.*) *and glucose* (100 *g/l.*)

Period of incubation (hr)	Glucose utilized (g/100 ml)	Mycelial felt (g/100 ml)	Fat produced		
			(g/100 ml)	(g/100 g of dry felt)	(g/100 g of glucose utilized)
24	1·16	0·32	0·04	12·5	3·4
48	2·73	0·68	0·19	27·9	6·9
68	3·49	0·80	0·40	50·0	11·5
96	3·50	1·00	0·48	48·0	13·7
120	4·10	1·17	0·54	46·1	13·2
144	4·70	1·28	0·60	46·0	12·8

Table 4. *Details of the growth of* P. spinulosum *in agitated and aerated culture in the metabolism solution from the experiment recorded in Table* 3, *fortified with essential inorganic salts* (*see text*)

Period of incubation (hr)	Glucose utilized (g/100 ml)	Mycelial felt (g/100 ml)	Fat produced		
			(g/100 ml)	(g/100 g of dry felt)	(g/100 g of glucose utilized)
24	0·95	0·42	0·03	7·1	3·1
48	2·74	0·90	0·25	27·7	9·1
70	3·72	1·00	0·39	39·0	10·5
90	4·63	1·13	0·66	57·4	14·2

The metabolism solution from the experiment recorded in Table 3 was used as the basis of the medium for a new experiment with *P. spinulosum*. The experiment recorded in Table 3 was stopped after 144 hr and the whole of the fermentation broth was run off. After the mycelial felt had been separated by filtration and centrifugation the filtrate, which contained 5·3 g/l. of residual glucose, was returned to the fermentor and fortified by the addition of $NaH_2PO_4.2H_2O$ (0·180 g/l.), $MgSO_4.7H_2O$ (0·060 g/l.), K_2SO_4 (0·055 g/l.) and NH_4Cl (1·600 g/l.). After the pH value had been re-adjusted with NaOH to 6·8 the medium was re-inoculated with 400 ml of a heavy suspension of spores of *P. spinulosum*. Incubation at 27° with stirring and aeration was then resumed. A higher rate of growth was now observed, for Table 4 shows that in 90 hr more fat (6·6 g/l.) was produced than was formed (6·0 g/l.) during 144 hr in the previous experiment (Table 3). The success of this experiment suggests that conditions might be found for using this mould in a process consisting of several stages, with periodical removal of the felt and reconstitution of the medium, up to a certain limit. The enhanced metabolic activity of the mould when grown from spores in the metabolism solution from a previous

experiment might have been occasioned, in part at any rate, by the presence of ready-formed metabolites essential for the initiation of growth, a subject to which reference has been made by Allen, Cooper & Rose (1954) in connection with the behaviour of certain bacteria.

Substantially improved results were obtained with *P. spinulosum* when the concentrations of $NaH_2PO_4.2H_2O$, $MgSO_4.7H_2O$ and K_2SO_4 in the medium were all raised by 50% while at the same time the concentration of NH_4Cl was doubled (Table 5). The high yield of fat, 11·4 g/l. with a fat coefficient of 12·0 in 162 hr, which was thus obtained, is somewhat in excess of the best yield (9·1 g fat/l., fat coefficient 11·8, in 192 hr) secured by Garrido *et al.* (1958) when they grew this mould in this same more concentrated mixture of nutrient salts in shaken flask cultures. In the fermentor, however, the glucose concentration was 10% (w/v), whereas Garrido *et al.* used 9% (w/v). This difference in concentration may have contributed to some extent to the difference in yield.

Table 5. *Details of the growth of* P. spinulosum *in agitated and aerated culture in a medium containing salts, glucose and ammonium chloride**

Period of incubation (hr)	Glucose utilized (g/100 ml)	Mycelial felt (g/100 ml)	Fat produced		
			g/100 ml	(g/100 g of dry felt)	(g/100 g of glucose utilized)
41	1·5	0·45	0·06	13·3	4·0
72	5·5	1·16	0·42	36·2	7·6
90	7·9	1·56	0·79	50·6	10·0
114	8·4	1·74	0·97	55·7	11·5
138	9·1	1·70	0·96	56·5	10·5
162	9·5	2·01	1·14	56·7	12·0

* The medium contained (g/l.): $NaH_2PO_4.2H_2O$, 0·270; $MgSO_4.7H_2O$, 0·090; K_2SO_4, 0·082; $ZnSO_4.7H_2O$, 0·013; $FeCl_3.6H_2O$, 0·040; NH_4Cl, 3·200; glucose, 100.

While the analytical data given in the Tables show correctly the course of sugar utilization and the formation of mycelial felt with its content of fat, they cannot be used to calculate the exact amounts of felt and of fat which have been formed at any given time, for two reasons. First, a certain amount of felt adheres to the walls of the fermentor and none of this is included in the samples withdrawn for analysis, and second, until an experiment is stopped it is not possible to ascertain the exact volume of fermentation broth in the vessel at any given time, because the quantity of sterile water brought in by the stream of air coming through the saturator tends to exceed slightly the quantity of water which escapes in the outgoing air. Generally, when using the fermentor, the yields of felt and of fat which have been isolated at the end of a trial run have proved to be somewhat in excess of those calculated from the analytical figures. In the experiment recorded in Table 3 the felt obtained when the contents of the fermentor were filtered amounted, when dry, to 270 g and contained 128 g of fat. The felt obtained when the experiment recorded

in Table 4 was stopped had a dry weight of 223 g and yielded 130 g of fat. Hence, the total dry felt obtained by combining the yields from these two experiments amounted to 493 g and the total quantity of fat was 258 g. These quantities were derived from the utilization of 1860 g of glucose in 234 hr. In the experiment detailed in Table 5 the felt formed by the utilization of 1900 g of glucose in 162 hr had a dry weight of 402 g and contained 228 g of fat.

Discussion

The present results, considered in conjunction with the data of Garrido *et al.* (1958), show that for the production of fat in glucose cultures developed with agitation and strong aeration, *P. spinulosum* is decidedly superior to *A. nidulans* and *P. javanicum*. Apart from the fact that *P. spinulosum* gave higher yields of fat than the two other moulds, it had the further advantage that it speedily lowered the pH value of the medium to about 2·2, thus rendering the solution highly resistant to air-borne bacteria. In none of the experiments with *P. spinulosum* was bacterial infection encountered and, in work with the two other moulds, serious infection was noted only in experiments which ran for more than six or seven days. In surface culture our strain of *P. spinulosum* produced citric acid to the extent of about 10% on the total sugar utilized and Dr. I. R. Shimi, working in these laboratories, found that this citric acid was gradually utilized as incubation proceeded. We have found, however, that citric acid cannot be detected in agitated, highly aerated cultures of *P. spinulosum*, and that the low pH value set up by this mould after about 24 hr of vigorous growth in these conditions is a result of the rapid utilization of the ammonium ion of the NH_4Cl. Nitrogen estimations on samples withdrawn from the fermentor showed that during the first 24 hr of incubation more than 95% of the available nitrogen of the medium was absorbed by the mould.

The yields of fat obtained in this enquiry by using *P. spinulosum* approach closely those secured by Enebo, Anderson & Lundin (1946), who cultivated *Rhodotorula gracilis* for the same purpose.

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Explanation of Plate

The fermentor employed, with vacuum pump and compressor.

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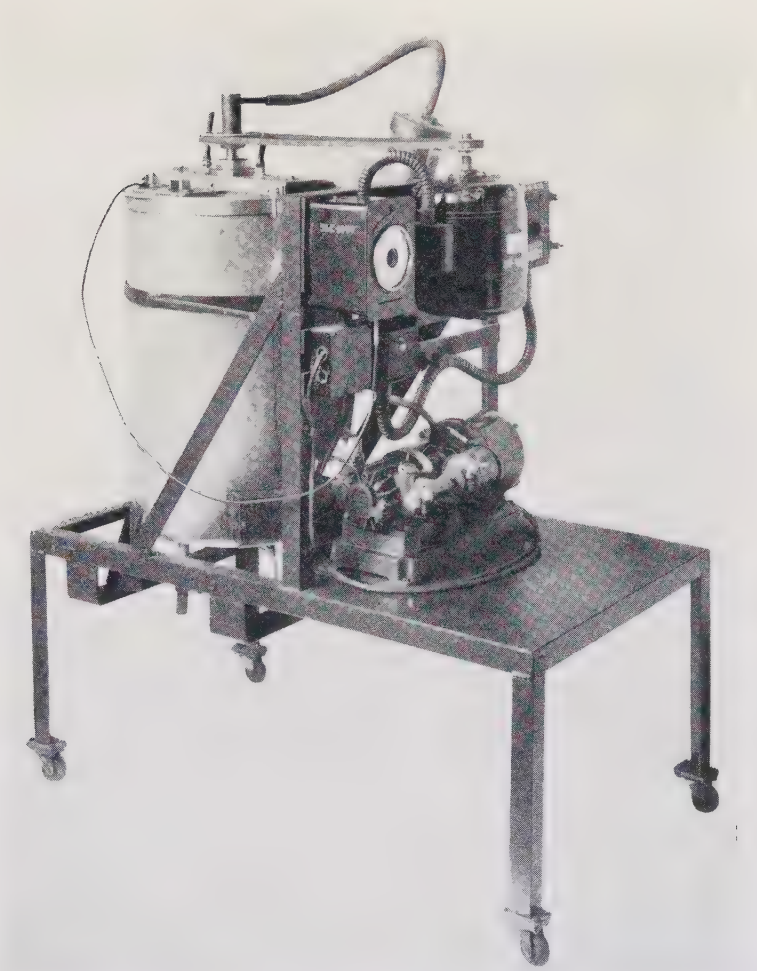

GARRIDO, J. M. & WALKER. T. K. — THE MYCOLOCICAL FORMATION OF FAT IN CULTURES GROWN WITH AGITATION AND AERATION. PLATE I

Millis, N. F., Husain, I., Hall, A. N. & Walker, T. K. (1958). *J. appl. Bact.* **21** (2), 299–307.

A MUCILAGE-FORMING *LACTOBACILLUS* SPECIES ISOLATED FROM CIDER. PART I. CHARACTERISTICS

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SUMMARY: A rod-shaped, facultative, heterofermentative lactic acid-forming micro-organism, isolated from cider, showed some similarities to *Lactobacillus pastorianus* van Laer, to *L. pastorianus* var. *brownii* and to *L. brevis* Orla-Jensen. Its most notable characteristic was an ability to form mucilage in a medium containing glucose, fructose or especially maltose. The highest levels of viscosity were obtained in a medium containing maltose (pH 5·1), after 12–16 days incubation at 25° under an atmosphere of carbon dioxide.

In common with many other fermented beverages, sweet ciders are susceptible to various types of disorder in which bacteria are the causative agents. Oiliness and ropiness are two manifestations of a disorder in which the liquid becomes viscous owing to the secretion of a mucilaginous material by bacteria growing in the cider. Whereas an oily cider has a viscosity comparable to that of a light lubricating oil, a ropy cider is highly viscous. Although spoilage of cider by an oily or ropy condition was recognized as a serious problem by Lloyd (1903), with the exception of a short paper by Grove (1917) and a recent communication by Carr (1957) there has been no original work published on this disorder of English ciders. Warcollier (1928), however, referred to this condition in French ciders, but apart from stating that a coccus forming long chains was present in oily cider, no further information was given. The description given by Grove (1917) of a slime-forming organism isolated by him from a ropy cider is also very incomplete. It was described as a non-sporing rod, occurring in pairs or short chains, showing revolving motility in a few cells. The Gram reaction was not recorded. The organism grew better in air than under anaerobic conditions, and slime formation in glucose-containing media was observed. This description would apply better to an *Acetobacter* species than to a member of the genus *Lactobacillus*, but in any case the evidence is too incomplete to permit a definite conclusion to be reached.

Rope-forming organisms have often been encountered in beer brewing operations and some of these contaminants have been shown to be *Acetobacter* species. Members of the family Lactobacteriaceae which can give rise to ropiness in beer wort, or in beer, include rod-shaped lactobacilli and also coccus forms. Most of these cocci have been assigned to the genus *Pediococcus* while others have been classified as *Streptococcus*

species (*cf.* Shimwell, 1947, 1949; Pederson, 1949; Kulka, Curtin Cosbie & Walker, 1949).

In the present paper the characteristics are described of an organism isolated from an oily cider at the Research Station, Long Ashton, Bristol.

Media and Methods

Media

The following previously described media were used. Apple juice (Millis, 1956); apple juice-yeast extract (Millis, 1956); malt wort medium (Tosic & Walker, 1946); pasteurized beer (Tosic & Walker, 1946) with the addition of glucose, 1% (w/v); yeast extract-peptone-glucose (YEPG) (Russell, Bhandari & Walker, 1954) and yeast extract-peptone (YEP) (Russell, Bhandari & Walker, 1954). Also pasteurized cider (sp.gr.1·03) was employed, and for studying the growth of the organism in milk a sample of skim milk (pH 6·5) was sterilized by heating in free steam for 30 min on 3 consecutive days.

The following media, details of which have not previously appeared, were also used.

Green malt extract (GME). This was prepared according to directions kindly supplied by Dr. M. Zakomorny in a private communication. Barley grains (620 g) were steeped in water for 6 hr. The excess water was then removed, the grains allowed to stand for 18 hr, and then again steeped in water for 6 hr. The barley, maintained in a moist condition, was then allowed to germinate and to grow until the plumules were about $2\frac{1}{2}$ times the length of the grains. The green malt was then minced, mixed with an equal quantity of cold water and the temperature of the mash raised to 45° at the rate of 1°/min. The mash was then allowed to stand for 30 min at 45°, heated to 70° at the rate of 1°/min, and maintained at 70° until 30 min after tests for starch had become negative. The mash was then filtered through muslin, the residue washed with water (1500 ml) at 70°, and the combined filtrate and washings boiled for $1\frac{1}{2}$ hr at such a rate as to cause a 10% loss of volume/hr. Following cooling and filtration, a final clarification was achieved by adding 5 g of egg albumen dissolved in 20 ml of cold water and heating at 100° in free steam for 20 min. The filtered green malt extract was adjusted to sp.gr. 1·04 and sterilized by free steam for 20 min on each of 3 successive days. Stock cultures were maintained in this medium. Reserve cultures were kept in the same medium in the presence of $CaCO_3$, in tubes which were sealed when evolution of CO_2 had ceased.

Yeast autolysate. Brewer's yeast (1000 g) was mixed to a cream with 1000 ml of water containing ethyl acetate (1%, v/v). The mixture was kept at 37° for 2 hr to allow frothing to subside. Toluene (1%, v/v) was then added with cautious shaking and the mixture was allowed to stand for 3 days at 37°. The dark brown supernatant fluid was then filtered clear and stocked in stoppered bottles.

Casein double digest-yeast autolysate (CDDYA). Casein double digest broth (Davis, 1939) was supplemented with yeast autolysate (5%, v/v). The nitrogen content of the medium was 5 mg/ml and the pH value was adjusted with HCl to 5·1–5·2. The medium was sterilized by free steam for 20 min on each of 3 successive days.

Solid media. Agar was used at 1·5% (w/v) for stab cultures and at 2% (w/v) for slant and plate cultures. Gelatin was used at the level of 15% (w/v).

Inoculation, incubation and reactions of the organism

Inoculation and incubation. Inoculations were generally made by a platinum wire loop from 84 hr cultures in medium GME. For the detection of acid and gas production from sugars and in controlled growth experiments a washed cell suspension was used as inoculum. An 84 hr culture in 10 ml of medium GME was centrifuged under aseptic conditions, the cells washed three times with sterile 0·9% (w/v) saline and finally resuspended in 10 ml of saline. Pasteur pipettes were used to transfer two drops (*c.* 0·1 ml) of this suspension to 6×1 in. culture tubes containing 20 ml of medium. In certain experiments a partially anaerobic atmosphere was obtained by evacuating vacuum desiccators, which contained the inoculated culture tubes, at the water pump (14 mm Hg) and then filling with CO_2. All cultures were incubated at 25° unless otherwise stated.

Production of acid and gas from carbohydrates. Seitz-filtered carbohydrate solutions were added to sterile medium CDDYA containing bromocresol green (0·0015%, w/v) and bromocresol purple (0·0015%, w/v); the test sugars were in 2% (w/v) concentration. Each tube of medium contained an inverted Durham tube and the cultures were examined daily for 20 days.

Optical form of the lactic acid formed from glucose. This was determined on a sample isolated as the zinc salt by the method of Pederson, Peterson & Fred (1926) from a 4 week culture in medium CDDYA plus 2% (w/v) of glucose and 3% (w/v) of $CaCO_3$.

Mucilage production in sugar media. Seitz-filtered solutions of L-arabinose, D-xylose, D-glucose, D-galactose, D-fructose, maltose, sucrose and dextrin were added to sterile medium CDDYA; the sugars were in 2% (w/v) concentration.

Tolerance to ethanol. These tests were made in medium GME containing absolute ethanol from 2 to 10% (v/v) in increments of 2%; sterile ethanol was added aseptically to the sterile medium.

Tolerance to hop antibiotic. The tests were made in medium GME containing pure humulone at concentrations from 0·00025% (w/v) to 0·004% (w/v), in convenient increments; sterile humulone in absolute ethanol was added aseptically to sterile medium GME. The effect of the ethanol addition was estimated by a control.

Liquefaction of gelatin. The medium was gelatin (15%, w/v) in medium YEPG.

Catalase reaction. The organism was grown on slants of malt wort agar medium containing sterile $CaCO_3$ (2%, w/v). At 1, 2, 3, 4 and 7 days, cultures were flooded with hydrogen peroxide (0·5N) and the evolution of oxygen bubbles looked for.

Production of ammonia: reduction of nitrate. The growth medium was YEPG plus KNO_3 (0·1%, w/v). After incubation for 10 days, Nessler's reagent was used to test for ammonia, while the α-naphthylamine-sulphanilic acid method was used to detect nitrite (Wilson & Miles, 1946).

Indole production. The medium was YEP plus DL-tryptophan (0·5%, w/v). The indole test was performed as recommended by Mackie & McCartney (1946), modified by shaking the culture with xylol instead of ether. The cultures were tested at 11 days.

Voges-Proskauer test. Following incubation for 10 days in medium YEPG, O'Meara's modification of this test (Mackie & McCartney, 1946) was used to detect the presence of acetylmethylcarbinol.

Analytical methods

Volatile acid. Five ml of the culture fluid were acidified with H_2SO_4 and steam-distilled in the presence of sodium tungstate and $MgSO_4$, the distillate being titrated against 0·1N $Ba(OH)_2$ to bromothymol blue.

Lactic acid. Estimations were made on 5 ml aliquots of culture fluid according to the procedure of Friedmann & Kendall (1929).

Total acid. Portions of 5 ml of culture fluid were either diluted to 30 ml with distilled water and titrated with 0·1N $Ba(OH)_2$ with bromothymol blue as indicator, or titrated with 0·1N NaOH using phenolphthalein. The values recorded are the means of estimations on triplicate tubes.

Growth determinations. For these, 3 ml of homogenized culture were diluted with 5 ml of water and the turbidity measured on a Hilger Spekker Absorptiometer equipped with neutral filters (Ilford H.508). The recorded values, expressed as the drum reading$\times$1000, are the means of triplicate tubes.

Viscosity. Measurements were made on Seitz-filtered culture fluid and recorded as the time of outflow (sec) through the standard capillary of an Ostwald No. 1 viscometer at 20°. The time of outflow (sec) of the uninoculated medium through the same viscometer was also measured and deducted from the experimental values. The recorded values are the means of estimations on triplicate tubes.

Results

Morphological characters

Shape, size, motility and Gram reaction of the cells. In medium GME the cells at 72 hr were uniform rods with dimensions 2·3$\times$0·8 μ. In hanging drop preparations mainly single cells were observed, but some short chains and pairs were also present. Motility was not observed. The Gram reaction was positive. Neither endospores nor capsules were detected.

Cultural characters

Single colonies on GME agar. At 7 days in an atmosphere of CO_2, the colonies were convex with entire margin, smooth and glossy in appearance and with a butyrous consistency. Growth was very slight under aerobic conditions.

Stab in GME agar. Growth was filiform. No surface growth was observed.

Stab in GME gelatin at 20°. Moderate beaded growth occurred with considerable branching along the line of inoculation. Neither surface growth nor liquefaction was noted.

Growth in medium GME. Growth was visible at 48 hr as a slight turbidity and a fibrous deposit. At 3 days the medium was highly viscous but at 3 weeks the viscosity was low and a semi-coherent viscid sediment was deposited.

Growth in pasteurized beer. Good growth was observed at 2 days, although this was not so strong as in medium GME. Viscosity and ropiness were present at 7 days.

Nutrient broth (pH 5·2). Growth was not visible at 3 weeks.

Apple juice. Growth was very slight in 10 days.

Apple juice-yeast extract. Growth was good, with ropiness and viscosity apparent at 3 days.

Pasteurized cider (sp.gr. 1·03). Growth was visible at 48 hr. At 7 days the cider became turbid and viscous but eventually the viscosity decreased, the cider became clear and a heavy flocculent sediment was deposited.

Litmus milk. No growth was detected after 3 weeks.

Physiological characters

Relation to temperature and oxygen. In medium GME the optimum temperature for growth was 25°. Slow growth occurred at 14–16° and at 38°, but there was no development at 43°. The organism was found to be a facultative anaerobe. The results of an experiment (Table 1) in medium CDDYA plus maltose (2%, w/v) showed that maximum growth and viscosity were obtained at 25° in the presence of CO_2.

Table 1. *The effects of temperature and atmosphere on growth, culture viscosity and acid formation by the cider lactobacillus in medium CDDYA plus maltose* (2%, *w/v*)

Period of incubation (days)	Temp. (°C)	In air			In CO_2		
		Viscosity (sec)	Growth*	Total acid†	Viscosity (sec)	Growth*	Total acid†
4	23	0	105	1·1		320	1·8
	25	1·8	115	1·49	10·3	570	2·3
	28	0	18	1·05	1·8	46	1·7
	30	0	10	1·58	3·3	52	2·0
8	23	2·2	108	1·45	5·0	418	2·0
	25	4·6	175	1·78	12·8	618	2·87
	28	6·8	58	1·3	10·0	106	2·0
	30	4·0	72	1·71	9·0	93	2·3
12	23	9·1	113	1·8	15·3	400	3·8
	25	19·2	280	2·0	29·0	690	4·15
	28	13·6	103	1·7	18·2	210	2·0
	30	13·0	128	1·9	18·0	200	2·1
16	23	13·0	180	2·1	14·0	420	2·7
	25	20·0	238	2·4	32·0	780	2·8
	28	13·0	140	2·4	15·0	248	2·8
	30	14·0	130	2·6	19·0	251	3·0
20	23	7·8	180	3·0	11·0	435	4·0
	25	18·0	242	3·0	23·0	620	4·8
	28	8·0	140	3·2	12·3	200	3·1
	30	10·0	132	3·4	17·3	230	3·2
24	23	3·3	171	3·4	10·0	380	3·4
	25	6·0	200	3·6	10·0	540	3·8
	28	6·0	150	3·7	11·3	180	3·8
	30	7·8	138	3·7	12·0	230	3·75

* As Spekker drum reading ×1000. † As ml of 0·1 N NaOH/5 ml of culture, using phenolphthalein.

Effect of hydrogen ion concentration. In pasteurized beer growth was strong in the pH range 4·3–5·9; no development occurred at pH 3·0 or pH 7·6. In medium CDDYA plus maltose (2%, w/v) in an atmosphere of CO_2, maximum viscosity occurred in

the pH range 5·1–6·0 (Table 2). The highest viscosity achieved in this experiment was at 12 days (pH 5·1).

Tolerance to ethanol and to humulone. The presence of 6% of ethanol (v/v) delayed the initiation of growth; with 10% (v/v) of ethanol growth was considerably reduced. No growth occurred in 10 days in the presence of 0·0005% of humulone (w/v) while 0·00025% (w/v) retarded growth.

Table 2. *The effect of the initial pH on the growth and culture viscosity of the cider lactobacillus in medium CDDYA plus maltose (2%, w/v) under CO_2*

Period of incubation (days)	pH 3·6		pH 5·1		pH 6·0		pH 7·1	
	Viscosity (sec)	Growth*	Viscosity (sec)	Growth	Viscosity (sec)	Growth	Viscosity (sec)	Growth
6	3·0	420	11·3	580	12·5	390	1·0	120
9	7·8	485	19·3	623	13·8	450	3·0	320
12	15·3	520	31·0	720	23·3	490	3·0	400
15	18·0	520	30·0	720	24·5	510	7·0	380
18	18·0	526	28·3	730	8·0	470	4·0	375

* As Spekker drum reading × 1000.

Biochemical characters

Production of acid and gas from carbohydrates. Acid was produced from L-arabinose, D-xylose, D-glucose, D-fructose, D-galactose and maltose, but not from sucrose, lactose, mannose, cellobiose, trehalose, raffinose, erythritol, mannitol, dulcitol,

Table 3. *The influence of different carbohydrates (2%, w/v) on growth, culture viscosity and acid formation by the cider lactobacillus in medium CDDYA under CO_2*

Carbohydrate	Incubation (days)	Viscosity (sec)	Growth*	Total acid†
Arabinose	3	2·5	440	1·5
	12	3·0	870	6·0
	24	0	700	6·3
Xylose	3	0	600	4·35
	12	1·0	785	8·2
	24	0	680	8·0
Glucose	3	1·0	122	0·1
	12	6·2	180	0·5
	24	1·3	130	1·0
Fructose	3	2·0	455	0·8
	12	2·8	640	3·2
	24	0	600	3·1
Galactose	3	0	380	3·0
	12	1·0	320	3·7
	24	0	380	3·6
Maltose	3	7·0	520	2·0
	12	22·0	700	2·0
	24	4·5	600	2·0

* As Spekker drum reading × 1000. † As ml of 0·1 NaOH/5 ml of culture, using phenolphthalein.

sorbitol, inositol, salicin, dextrin, soluble starch or inulin. Although gas production in Durham tubes was not observed, the heterofermentative nature of the organism was revealed by the finding that the ratio of lactic acid to volatile acid produced in medium CDDYA plus glucose (3%, w/v) at 14 days was 100:38. DL-lactic acid was isolated from a culture on a glucose medium containing $CaCO_3$.

Mucilage production in different media. The data obtained (Table 3) show that while glucose and fructose caused small increases in viscosity, by far the highest viscosities were attained in the presence of maltose. Media containing sucrose or dextrin supported only a trace of growth, without the production of viscosity or acidity.

Additional findings. The organism did not produce ammonia, indole, acetylmethylcarbinol or nitrite. Tests for catalase and gelatin liquefaction were negative.

Discussion

The characters of the organism show that it belongs to the Tribe Lactobacilleae Winslow *et al.* of the Family Lactobacteriaceae Orla-Jensen in the order Eubacteriales (Buchanan). Further differentiation according to Bergey's Manual of Determinative Bacteriology (Breed, Murray & Hitchens, 1948) places the organism in the genus *Lactobacillus* Beijerinck. However, there does not appear to be a really close resemblance to any of the *Lactobacillus* species listed by Breed *et al.* Further, the organism does not appear to be identical with any of the *Lactobacillus* species isolated from beer (Bhandari, Russell & Walker, 1954; Shimwell, 1949). The organism, however, does show some similarity to *L. brevis* (Orla-Jensen) Bergey *et al.*, to *L. pastorianus* (van Laer) Bergey *et al.* and to *L. pastorianus* var. *brownii*. The latter is a rope-forming organism isolated from beer (Shimwell, 1949). Allowing for the rather incomplete descriptions of *L. pastorianus* (van Laer) Bergey *et al.* and *L. pastorianus* var. *brownii*, the cider lactobacillus seems to resemble *L. brevis* most closely. In terms of carbohydrate fermentation the cider lactobacillus is quite similar to *L. brevis* (Orla-Jensen) Bergey *et al.*, which is partly characterized by its failure to produce acid from raffinose, lactose, mannitol and sucrose. It is, however, important to note that many strains of *L. brevis* characterized by Rogosa *et al.* (1953) were able to ferment these sugars.

The principal differences between our description of the cider lactobacillus and the description of *L. brevis* in Bergey's Manual of Determinative Bacteriology are in optimum temperature (25° against 30°), in the production of mucilage by the cider organism in certain media and in the ability of *L. brevis* to develop in milk and to utilize lactate as the sole source of carbon.

Recently we submitted the cider lactobacillus to Dr. M. E. Sharpe for serological examination, and she found that the organism reacted specifically with the group E (Sharpe & Wheater, 1957) serum for lactobacilli; this shows our strain to be a serotype of *L. brevis*. The extensive studies made by Sharpe (1955) suggest that many strains of *L. pastorianus* should be placed with *L. brevis*, on the basis of their sharing of common antigenic components.

The most notable characteristic of the cider lactobacillus is undoubtedly its ability

to produce mucilage in suitable media containing glucose, fructose or, especially. maltose. Of particular interest is the failure of the organism to utilize sucrose for growth and for the production of mucilage, since this sugar is readily converted into dextran by certain *Leuconostoc* species and into levan by some *Aerobacter* and *Bacillus* species (Hehre, 1951).

Subcultures of the cider lactobacillus have been lodged at the Research Station, Long Ashton, Bristol, at the National Institute for Research in Dairying, Shinfield, Reading, and at the National Collection of Industrial Bacteria, The Chemical Research Laboratory, Teddington, Middlesex.

One of the authors (N.F.M.), who held the Boots Research Scholarship at the Agricultural and Horticultural Research Station, Bristol, during this investigation, has much pleasure in acknowledging the help of Professor T. Wallace and his colleagues at the Station. The authors are grateful to Dr. E. M. Sharpe of the National Institute for Research in Dairying, University of Reading, for undertaking the serological examination of the organism.

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THE CONSTRUCTION OF BIOLOGICAL FILM IN A PERCOLATING SEWAGE FILTER

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SUMMARY: On a microscope slide arranged to simulate a percolating sewage filter the sequence of invading organisms was examined and a primary and secondary invader recognised. These organisms form a substratum suitable for fungal growth.

This is an attempt to obtain a general picture of the ecology of a percolating sewage filter, both as regards the prisere, the first natural succession of biological types to colonise a habitat, and the climax community.

Apparatus and Methods

Early seral communities. To study these, direct microscopical examination and cinephotomicrography were used. The apparatus, shown in Plate 1, contained a phase contrast microscope and the image, produced by a ×40 objective, was either observed through the usual ocular system or projected through a special lens (×1/3 photographic, E. Leitz, Ltd.) to form a real image on the photographic emulsion in the 35 mm motion picture camera above the microscope. To compensate for small changes in the focal length of the objective caused by room temperature variations, a small steel bracket was attached to the body tube of the microscope so that the objective mount was supported by a short length of $\frac{3}{4}$ in. diameter Perspex tube (Plate 1). The length of the tube was adjusted by trial and error so that the objective was moved by thermal expansion and contraction of the Perspex to and from the object plane sufficiently to compensate exactly for changes in focal length.

In the object plane was placed a small glass 'microfilter' to simulate the surface of medium in a percolating filter. It consisted of a 3×1 in. microscope slide to which were cemented two thin strips of glass covered by a $2 \times \frac{7}{8}$ in. no. 0 coverglass cemented in position. Glass rings were cemented over each open end of the channel to form reservoirs for the addition and removal of the fluid environment (see Fig. 1).

The fluid was delivered to the inlet reservoir by an air lift which, together with the impingent air jet over the reservoir, kept the fluid agitated and aerated. The rate of flow through the microfilter was in all experiments about 100 ml/hr and was determined by the head of fluid in the reservoir, which in turn was controlled by an electromagnetic valve (Londex type LF/V, Anerley Works, London, S.E.20) operated

through a proximity switch (Tektor, Fielden Electronics Ltd., Buckingham St., London, W.C.2) with electrodes in the reservoir. This rate gave a mean linear flow through the channel of 5 cm/sec, which is roughly that at which sewage flows over new medium in a percolating filter. The liquid passing out of the microfilter was removed from the effluent reservoir through a measuring flask by a Geissler pump.

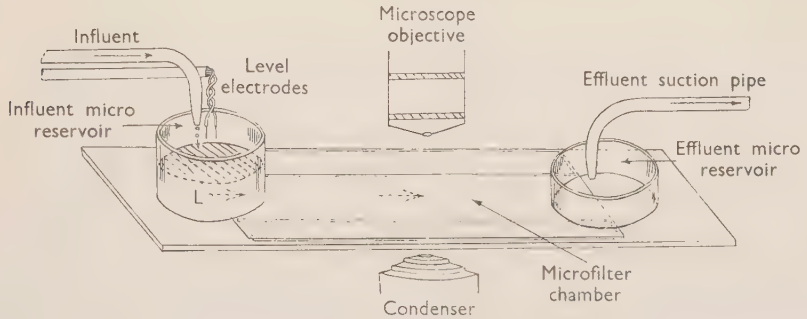

Fig. 1. Diagram of microfilter used for observing growth of film.

The Leitz Heine condenser used allowed phase contrast observations to be made at any depth in the channel. Three forms of illumination were provided: normal tungsten filament, a 150 J point source xenon discharge tube (Type F.A.5 Mazda) and a high pressure point source mercury vapour tube (Type MED/250 Mazda). The tungsten and xenon sources were coupled to the camera and to a timing circuit so that regular exposures were made automatically. At 100 J the flash duration of the xenon tube was less than 1000 μsec. The xenon flash tube was used for observations on total populations, including bacteria suspended in the sewage and thus moving in the field. A 1 sec exposure with the tungsten lamp recorded only those organisms which were relatively motionless, and thus selectively recorded organisms anchored to the internal surface of the filter channel. A series of photographs obtained by this method can be projected as a time lapse motion picture, and much of the information was thus obtained. The mercury vapour lamp was used with a Woods filter to provide light of wavelength 366 mμ for fluorescence microscopy.

Later seral communities. These were examined by preparing stained radial sections of biological film taken from a working percolating filter at a sewage works. Biological film was allowed to develop on blocks of paraffin wax placed on the surface of the filter. At intervals a block was removed and immediately fixed in Meves's weak fixing solution (Gatenby, 1950) for 3–4 days at room temperature. After fixing, the specimen was washed in running water for 1 day and then progressively dehydrated in alcohol and cleared in xylol, the wax core being simultaneously dissolved. After embedding in paraffin wax (congealing point 49·5°), sectioning, and dewaxing, the sections were stained in 0·5% Chlorazole Black E dissolved in pyridine (Cannon, 1937, 1941; cited by Gatenby, 1950) and differentiated in clove oil. The preparation was mounted in 'Cristalite' thinned with xylol. Many fixatives, stains and mounting media were tried but this technique was the only one satisfactory. Above average

care was needed as the material was more delicate than most normal tissue. For control purposes pure cultures of fungi were grown on asbestos pads irrigated intermittently with sterile Lab-Lemco broth, in a sterile vessel at 25°.

Results

Prisere

Direct observation and timing of small suspended particles crossing the field at various depths showed that laminar flow occurred in the filter channel. The liquid in contact with the surfaces remained almost at rest. Flow rates up to 10 cm/sec were observed in the middle of the channel. Suspended particles remained at an even depth with little tendency to settle in the length of the channel.

To confirm that there was adequate aeration within the cell, dissolved oxygen measurements were made by the modification of Winkler's method introduced by Fox & Wingfield (1938) on influent and effluent solutions of peptone (0·01%) in which organisms were growing rapidly. These tests always indicated values greater than half-saturation for the bulk effluent, but did not demonstrate that aerobic conditions necessarily existed in the almost motionless layers of liquid.

When settled domestic sewage such as is normally treated on percolating filters at a sewage works was strained through muslin to remove large particles and passed through the microfilter, organisms were soon deposited on the upper and lower glass surfaces of the filter. It was considered that the lower surface was the better approximation to the surfaces in a real filter and observations were therefore limited to that surface. Presumably because of an unsuitable environment for growth, many of the organisms first deposited degenerated quickly and were lost. Sometimes they remained in position for long periods without movement or growth. After 3–4 hr several small organisms (primary invaders, Plate 2) had invariably been deposited at different points in the field and these divided at once with little or no subsequent migration of the daughter cells. It was repeatedly observed in different experiments that these pioneer communities were always composed of organisms of identical morphology. They continued to multiply to form flat colonies in contact with the surface and rarely more than one organism thick. Although loose at first their structure rapidly became more compact. Shortly after the deposition of these organisms another much larger organism always appeared (secondary invaders, Plate 2). In contrast to the first, although in contact with the glass surface, it did not adhere firmly to it but migrated slowly downstream, dividing as it went, until one organism came into contact with a group of primary invaders. Here the drifting organism became firmly held and, being larger and dividing more rapidly, gave rise to a colony which soon tended to extend beyond the limits of the original consociation. Then, unable to resist even the slow flow of the surrounding medium, its outer and upper edges were constantly eroded. The resulting association enlarged and either joined with one of the many similar surrounding associations or, having attained a certain diameter (20–30 μ), started to trap solid material from the passing sewage, including other bacteria whose fate within the association was not observable with the phase contrast microscope. Plate 2 shows a typical succession of stages.

By 8–10 hr the community was too dense to allow either the free passage of sewage or proper observation. Motile protozoa could be seen after 10 hr and amoebae appeared on the glass surface an hour or two later.

The mean generation time of the pioneer organisms was 104 min and that of the secondary invader was 94 min. One secondary invader divided after only 18·5 min, while one of its granddaughters took 186 min to divide. In one experiment the range of doubling times of the primary invader was 79–136 min.

To determine the fate of organisms of other types trapped in the associations, strains of *Escherichia coli* and *Staphylococcus aureus*, and a filamentous organism isolated from sewage, were grown on nutrient agar containing 0·01% Fluolite T.P. 180 (44·7% pure; I.C.I., Ltd.). Even in much higher concentration this substance did not affect the growth, appearance, or biochemical reactions of the organisms used but imparted to them even at low concentration a brilliant blue fluorescence in light of wavelength 366 mμ. Two drops of a washed suspension in distilled water (Brown's opacity 10) of one of these organisms was added every 2 min for 20 min to the sewage in the influent reservoir of a microfilter which had been operating for 6 hr. When occasionally observed by ultraviolet light, the organisms were seen not to enter the associations but to remain on the surface, usually at the edge of an association, where they gave rise to small fluorescent clones.

Climax community

Plate 3 shows the increase in film thickness on the surface medium of a percolating filter during the period December, 1955–May, 1956. At first only an amorphous mass was to be seen, but later a definite structure was evolved consisting of alternating tangential and radial layers of hyphae of *Sepedonium*. Examination of the basal layer failed to yield much information concerning its origin. It appeared to consist of fine silt or mucilaginous material mixed with hyphae of fungi other than *Sepedonium*. These no doubt are the remains of earlier invaders previously described by Tomlinson (1941, 1946). In the basal layer the only genus which could be identified with reasonable certainty was *Phoma*.

The cell walls of the inner layer were well formed in the first sections but degenerated during the following spring with increasing film thickness. No bacteria could be demonstrated microscopically to be associated with this degeneration or with the normal structure of the middle layers, but it is doubtful whether free bacteria within the film would have remained in position during embedding. Throughout the remaining layers the film consisted of almost a pure culture of *Sepedonium* with the rare appearance of a protozoon. The layered structure of the *Sepedonium* was also seen in control cultures grown in the laboratory as described.

Discussion

We have considered in a microfilter an invasion of virgin medium which occurs but once in the life of a percolating filter, but the annual sloughing process is so complete that it is probably more akin to a complete catastrophe than to vernal change and the annual pseudosubsere produced should closely resemble the prisere. For this

reason it seemed permissible to follow the prisere as far as possible in a microfilter and complete the observations on a virgin surface placed on a filter which had been in operation for many years.

Different sewage works produce grossly different climaxes which we have been unable to consider, neither have we considered edaphic differences presented by wax, glass and more usual filter media such as gravel and coke.

The reason for the layered structure is not clear. It occurred similarly in the laboratory, where flow rate, temperature and composition of the medium were constant. Nothing was seen to suggest that the tangential layers were associated with reproductive bodies.

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Explanation of Plates

Plate 1. The apparatus used for observing the growth of bacteria on a glass 'microfilter' simulating the material in a percolating sewage filter.
1, optical tube clamp; 2, steel bracket; 3, Perspex expansion tube; 4, dove-tail slide; 5, optical tube; 6, microscope body; 7, parfocal focussing telescope; 8, objective ($\times 40$ phase contrast apochromat); 9, microfilter.

Plate 2. Photographs ($\times 1000$) of growing bacteria on the microfilter, showing the stages of development of film. Taken with a 5 sec tungsten filament lamp exposure, to show only those organisms adhering to the lower glass surface. The arrows show examples of the secondary invaders. The smaller organisms are all primary invaders.

Plate 3. Radial sections of biological film taken from a percolating sewage filter treating town sewage, showing increasing complexity with age.
(*a*) December, 1955. Loose material forming beginnings of new film.
(*b*) February, 1956. Film consists mainly of compact mycelium of *Sepedonium*.
(*c*) April, 1956. *Sepedonium* becoming thicker.
(*d*) May, 1956. *Sepedonium* reaching maximum thickness and showing alternate radial and tangential layers.

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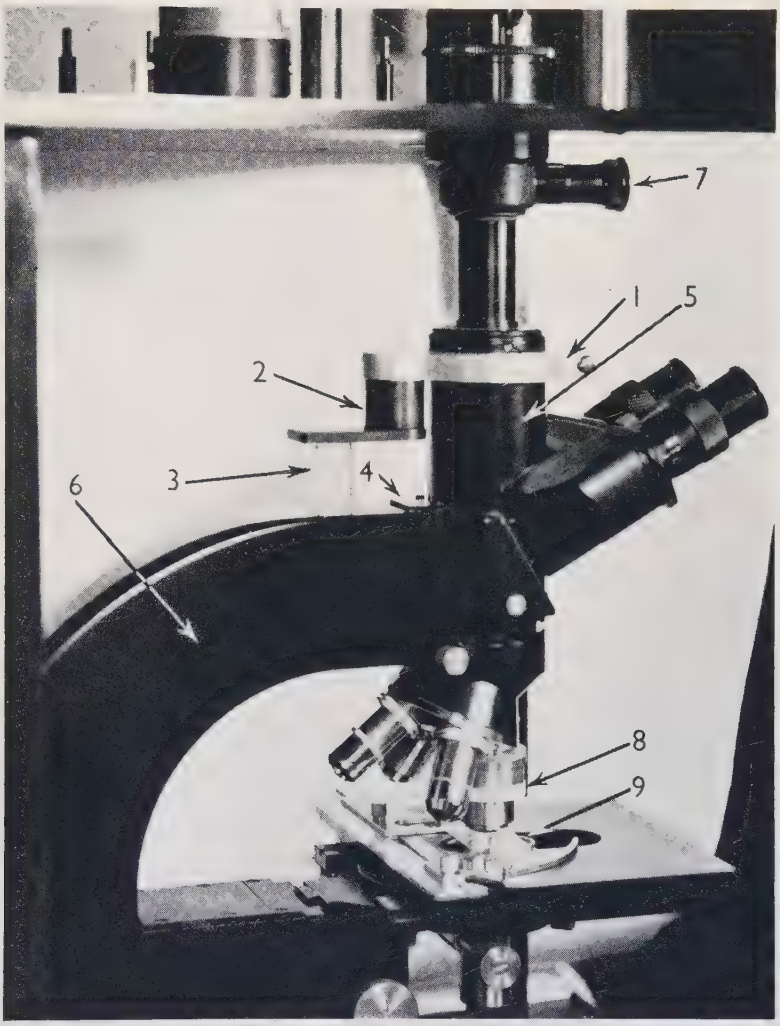
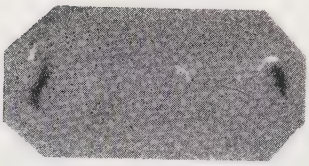


G. C. Ware & J. E. Loveless — The Construction of Biological Film in a Percolating Sewage Filter. Plate 1



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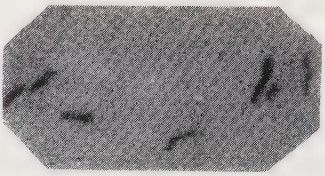

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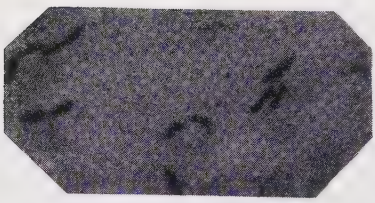
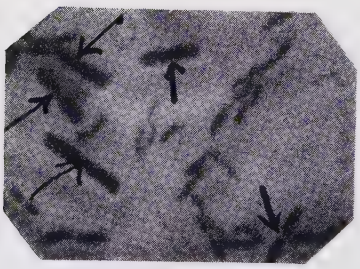


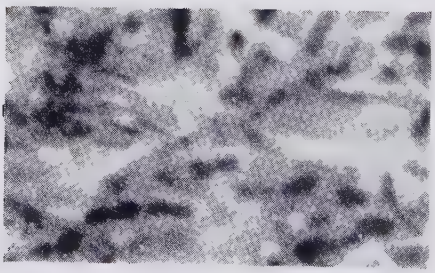
6 hr

8 hr



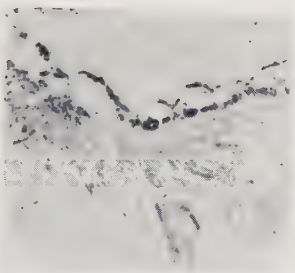
10 hr

11 hr



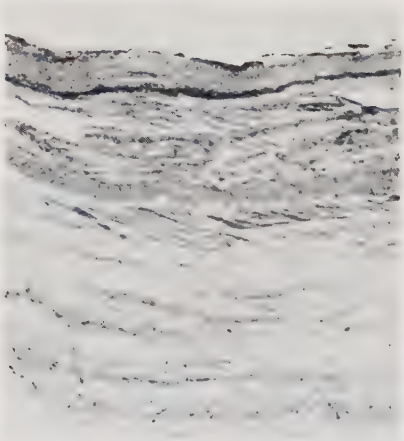
12 hr

G. C. Ware & J. E. Loveless — The Construction of Biological Film in a Percolating Sewage Filter. Plate 2



(*a*)

(*b*)



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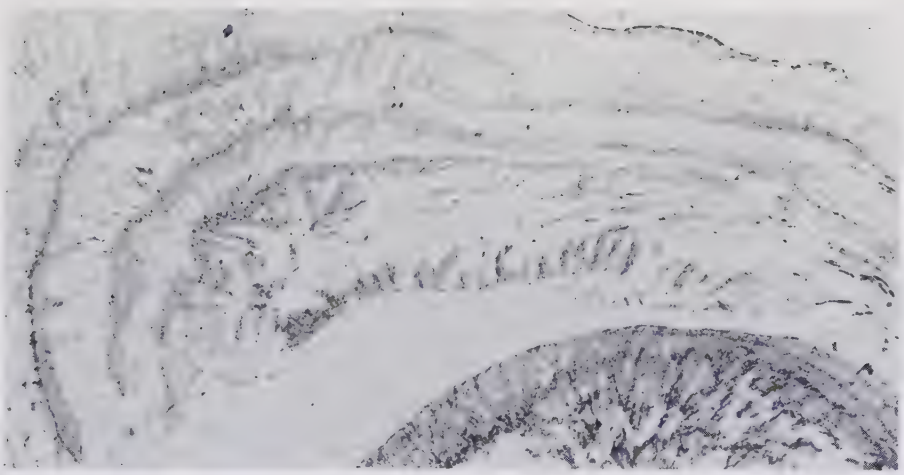

(*d*)

G. C. Ware & J. E. Loveless — The Construction of Biological Film in a Percolating Sewage Filter. Plate 3

Barnes, E. M. & Shrimpton, D. H. (1958). *J. appl. Bact.* **21** (2), 313–329.

THE EFFECT OF THE TETRACYCLINE COMPOUNDS ON THE STORAGE LIFE AND MICROBIOLOGY OF CHILLED EVISCERATED POULTRY

By ELLA M. BARNES and D. H. SHRIMPTON

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SUMMARY: Chlortetracycline, oxytetracycline and tetracycline, incorporated at 10 p/m in the ice slush used for cooling, were equally efficient in extending the storage life of eviscerated poultry which was subsequently frozen, thawed and stored at 1°. There was complete correlation between the sensory assessment of 'off' odour and the total numbers of micro-organisms present when these were determined by taking bulk samples of skin from the area under the wing and of the surface tissue of the visceral cavity near the vent. Direct microscopical counts confirmed that the latter area was the most heavily contaminated part of the visceral cavity. Non-pigmented strains of *Pseudomonas* were the main spoilage organisms of untreated chickens but *Achromobacter* strains and yeasts were responsible for the spoilage of the chlortetracycline treated chickens. Fluorescence was only observed in spoiled chickens where pigmented strains of *Pseudomonas* occurred in significant numbers. Spoilage in both control and antibiotic treated poultry was accompanied by a rise in pH in the contaminated muscles, but for various reasons this change could not be used as a direct measure of the extent of the microbial contamination.

Both chlortetracycline and oxytetracycline are now being used in the United States of America, Canada and some European countries as preservatives for extending the short storage life of chilled eviscerated poultry. Many comparisons of the effectiveness of these antibiotics as preservatives of eviscerated poultry have been made in the U.S.A., where eviscerated poultry intended for the 'fresh' market is chilled continuously through all stages of distribution to the consumer. In the United Kingdom where the broiler industry is only newly established, methods of distribution are quite different. Here, increasing numbers of eviscerated broiler carcases which are intended for the 'fresh' market are frozen immediately after processing, and distributed in either unrefrigerated or refrigerated transport to the retailer and thawed before resale to the consumer.

It was decided to compare the effectiveness of chlortetracycline, oxytetracycline and tetracycline for preserving poultry processed under British conditions and to study the changes in the numbers and types of spoilage organisms present. Because the storage life is longer the lower the temperature, it was considered that any differences would be magnified if a temperature near to 0° were chosen for holding the carcases. Accordingly 1° was used, but to simulate some commercial holding conditions more closely a holding temperature of 5° was also employed.

In attempts to assess the degree of spoilage of chilled eviscerated poultry, determinations of total numbers of micro-organisms present have often failed to correlate with an assessment based on sensory examination (e.g. Vaughn, Nagel, Sawyer & Stewart, 1957). Many factors may be involved: the metabolic products of different

spoilage floras, the method and site of sampling and the method of sensory examination. Little is known of the first of these. Of the various sampling methods available, the one most frequently used when examining antibiotic-treated poultry is to rinse a weighed chicken in an equal weight of sterile distilled water. The numbers of organisms/g of chicken are then regarded as equivalent to the number/ml of rinse water (Kohler *et al.* 1956–7). Ayres *et al.* (1956) swabbed known areas of chicken, and showed that this method gave larger numbers than rinsing. A method used more frequently in meat examinations is to excise a known area to a depth of a few millimetres and then to prepare a homogenate of the sample (Haines, 1931). In the experiments described below a new sampling method has been used successfully, as well as the more traditional methods.

The choice of sampling sites on a poultry carcase is difficult, and may partly depend on whether one is specifically looking for faecal contamination or for the psychrophilic spoilage organisms which will cause the most harm to poultry stored at chill temperatures. Ziegler, Spencer & Stadelman (1954) showed that the area of skin immediately below the wing was usually heavily contaminated but no figures have been found for different areas within the visceral cavity. An investigation of the distribution of bacteria and yeasts within the visceral cavities of stored poultry carcases is described in this paper.

Sensory evaluation is necessarily subjective, so that comparisons between different standards of assessment are not possible.

Materials and Methods

Birds, and preparation of poultry

Birds. The birds were reared intensively on deep litter under standard conditions and were fed on a diet containing a supplement of chlortetracycline at 5 p/m. They were delivered to the packing station on the morning of the experiment when they were 11 weeks old.

Procedure at packing station. The birds were stunned electrically, killed with a knife and bled. They were then immersed in turbulent water at about 50° for 2 min (slack-scalded), and also the hocks and neck of each were immersed in turn in water at about 60° for $\frac{1}{4}$ min (sub-scalded). The scalded birds were 'wet-plucked' mechanically, eviscerated and washed, the entire procedure from stunning to final washing taking about 20 min.

Application of antibiotics. Solutions of chlortetracycline, oxytetracycline and tetracycline were added to the ice slush used for cooling the freshly eviscerated birds, to an initial concentration of 10 p/m, in the manner previously described for chlortetracycline (Shrimpton, 1957), separate tanks being used for each antibiotic and for the control group where no antibiotic was added.

Transport of the poultry. When the chickens had been chilled and trussed they were individually wrapped in polythene bags and frozen overnight in a blast freezer where the air temperature was −26°. On the following morning the frozen chickens were removed from the freezer, brought to the laboratory by road, the total time without refrigeration being 12 hr, and stored in constant temperature rooms.

Storage and sensory examination

Storage. Nine chickens from each group were held at 1° and a further ten from each of the control and chlortetracycline-treated groups were held at 5°. All remained in their original polythene wrappers throughout the storage period.

Sensory examination. Each chicken was examined daily by two observers, care being taken to handle only the polythene and not the chickens themselves. When both observers first recorded a change in smell, the carcase was considered to have a slight 'off' odour. Two subsequent stages were defined as an 'off' odour and a strong 'off' odour. Because of the subjective nature of observations of this type, the first and last stages of deterioration have always been recorded. A statistical analysis has been made only for the first stage of deterioration, because some chickens were removed for bacteriological examination during subsequent stages of spoilage.

Chemical examination

Determination of pH. About 1 g of muscle was homogenized with 10 ml of distilled water in a Marsh-Snow blendor (Marsh & Snow, 1950) and the pH of this homogenate was determined using a glass electrode and a direct reading pH meter.

Detection of fluorescence. After the visceral cavity was opened (see sampling method 3, below) the carcase was placed about 6 in. below an ultraviolet lamp (Osram MBM/V, a 125 W mercury discharge unit enclosed in a bulb of Woods glass). The position, colour and intensity of the fluorescent regions were noted.

Sampling

Three methods were used.

In method 1, cotton wool swabs were rubbed over 50 cm^2 of chicken, a metal frame being used to define the area. The swabs were immediately suspended in 10 ml of 0·1% peptone water (Walker & Ayres, 1956; Straka & Stokes, 1957), cooled in ice slush, and the bacteriological examination made within 6 hr.

In method 2, which was devised in collaboration with Dr. Margaret Thornley, pieces of skin were removed aseptically from under the wing until a total weight of 2 g was obtained. Thin slivers from the surface of the visceral cavity totalling 3 g were also removed, working from the vent inwards. These were bulked with the skin sample and the whole homogenized with 45 ml of quarter-strength Ringer's solution to give the first dilution. The combined sample represented an area of approximately 50 cm^2 of the original surface.

In method 3, the visceral cavity was opened by cutting through the breast bone, using sterile instruments and leaving the chicken on its polythene wrapper. Samples of the peritoneal surface were removed from all sites which were fluorescent and from certain other selected sites (Fig. 1). Following the method of Haines (1931), thin slices of tissue of surface area 0·5 cm^2 were excised with a sterile scalpel, using a wire frame as a guide. When muscle was removed from over an air sac, the thin wall of the sac was supported from within with a pair of scissors. Finally, samples were removed from the deep muscle tissue using sterile instruments, but as no attempt was made to sterilize the infected surface layer some carry over of organisms

might be expected. Each sample was mechanically shaken with 10 ml of sterile saline (0·9% w/v) in a 1 oz bottle containing glass beads.

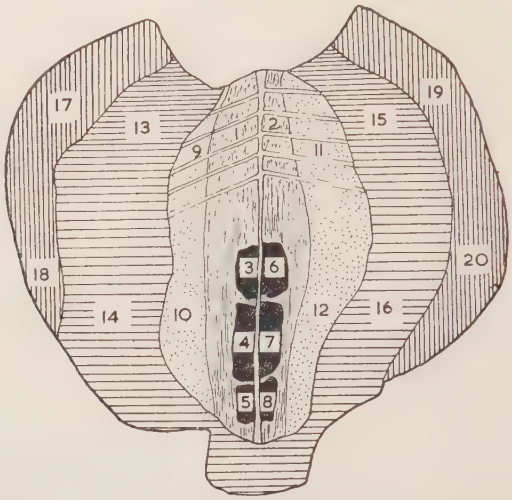

Fig. 1. The regions of the visceral cavity and the pectoral muscle which were sampled for direct microscopical counts. Regions 1 and 2, lungs; 3–8, kidneys; 9, 11, 13, 15, thoracic muscles; 10, 12, 14, 16, abdominal muscles; 17–20, pectoral muscles.

Bacteriological examination

Direct microscopical count. For obtaining the total numbers of bacteria, 0·01 ml amounts of suitable dilutions of suspensions prepared by method 3 were spread evenly over 1 cm^2 on a microscope slide, Gram-stained, and the numbers estimated microscopically from 20 fields counted by two observers. For yeasts, a few drops of the suspension were placed in a Thoma counting chamber (Hawksley, London) and the cells counted using phase contrast illumination. A budding yeast cell was counted as a single unit.

Colony counts at 1°, 20° *and* 37°. Decimal dilutions of the sample were prepared in quarter-strength Ringer's solution and 0·025 ml of each dilution was spread over one-quarter of a Petri dish containing the required agar medium. Difco heart infusion agar was used throughout, but for the 1° and 20° counts duplicate plates were also prepared using Tryptone soya agar (Oxoid) and Hartley's digest agar for alternate samples. As there was little difference in the counts obtained on the three media, average results have been recorded. The plates were incubated for 2 weeks at 1°, or for 3 days at 20° or for 1 day at 37°.

Faecal streptococci. These were counted on thallous acetate tetrazolium agar (pH 6·0) by the method of Barnes (1956).

Yeasts. The modified yeast salt agar of Davis (1958) was used and the plates were incubated for 4 days at 20°.

Identification of micro-organisms. Of the bacteria, only those growing at 1° were studied. To determine the distribution of types, all the colonies from the highest dilution were examined and where there were less than 5 of these a proportion of

the colonies from the lower dilution was also selected. Methods worked out by Shewan, Liston & Hodgkiss (pers. comm.) were used to distinguish the various Gram-negative organisms, in particular the pigmented and non-pigmented pseudomonads and achromobacters. The criteria included morphology, motility and type of flagellation, sensitivity to penicillin and oxytetracycline (Shewan, Hodgkiss & Liston, 1954), pigmentation (using the medium of King, Ward & Raney, 1954 for fluorescin production), oxidative or fermentative ability (Hugh & Leifson, 1953), oxidase reaction (Kovacs, 1956) and behaviour in litmus milk. No attempt was made to name individual species. The tests in the Hugh & Leifson medium and in litmus milk were done by Dr. B. P. Eddy and the results of his study of the non-motile organisms (mainly *Achromobacter*) are to appear later.

A detailed study is being made by Dr. M. Ingram of the types of yeast present: this will be reported elsewhere.

Results

Comparison of methods and sites of sampling

The chickens used in the storage experiments described below were taken from the packing station at the same time as a microbiological survey was being made. This survey consisted of taking swabs of both poultry and equipment at various stages of the process.

Table 1. *The numbers of micro-organisms obtained by two sampling methods from washed eviscerated poultry before cooling*

Method*	Carcase		Colony count at 1°				Colony counts (no./cm²) at		Faecal strepto-cocci
			No.	Proportion (%) of					
	No.	Site	(/cm²)	A†	B†	C†	20°	37°	(no./cm²)
1	1	Outside	$1{\cdot}2\times10^{2}$	44	45	11	$3{\cdot}0\times10^{3}$	$1{\cdot}5\times10^{3}$	$9{\cdot}4\times10^{1}$
		Inside	$2{\cdot}8\times10^{2}$	50	50	0	$4{\cdot}2\times10^{4}$	$3{\cdot}0\times10^{3}$	$4{\cdot}3\times10^{2}$
1	2	Outside	$2{\cdot}8\times10^{2}$	44·5	44·5	11	$1{\cdot}6\times10^{3}$	$5{\cdot}2\times10^{3}$	$1{\cdot}7\times10^{2}$
		Inside	$2{\cdot}0\times10^{2}$	75	0	25	$1{\cdot}3\times10^{4}$	$1{\cdot}7\times10^{3}$	$4{\cdot}4\times10^{2}$
2	3		$1{\cdot}9\times10^{3}$	0	100	0	$7{\cdot}5\times10^{4}$	$3{\cdot}7\times10^{4}$	$2{\cdot}4\times10^{3}$
2	4		$9{\cdot}8\times10^{3}$	0	75	25	$2{\cdot}1\times10^{5}$	$3{\cdot}4\times10^{4}$	$1{\cdot}2\times10^{4}$
2	5		$3{\cdot}3\times10^{3}$	17	83	0	$4{\cdot}9\times10^{4}$	$2{\cdot}8\times10^{4}$	$2{\cdot}6\times10^{3}$

* Method 1, surface swabbing. Method 2, homogenization of combined skin and visceral cavity tissue.

† A, *Pseudomonas* strains (pigmented and non-pigmented); B, *Achromobacter* strains; C, miscellaneous organisms (*Cytophaga* and *Flavobacterium* strains and organisms related to coli-aerogenes bacteria).

In order to relate the swabbing method (method 1) to the sampling technique used in the storage tests (method 2), chickens were sampled immediately after the last wash (before cooling in ice slush) and tested by both methods. Swabs were taken of approximately 50 cm² of the breasts of two carcases and the visceral cavities were also tested, although with only a small hole at the vent this was difficult. It was estimated that this area also was about 50 cm², although it was possibly greater. Three other chickens were sampled by method 2 described above. The sample consisted of 2 g of skin taken from under the wing and 3 g of surface tissue from the

anal end of the visceral cavity. These areas were chosen as they were considered to be the most heavily contaminated and therefore the first to cause any 'off' odour. Their combined area was also about 50 cm^2. The results, given in Table 1, suggest that a proportion of the organisms was not recovered by swabbing. Only the psychrophilic organisms were examined for any differences in the proportions of types obtained by the two methods: the higher psychrophilic count found by method 2 was accounted for by *Achromobacter* spp., some of which formed very gelatinous colonies. This property may have caused a proportion of them to fail to be removed from the swabbed surface.

Comparison of chlortetracycline, oxytetracycline and tetracycline treated poultry stored at chill temperatures

Two experiments were carried out at the same time on poultry processed together, so that the conditions were strictly comparable. In the first experiment the effects of 10 p/m of each of the three antibiotics on poultry held at 1° were compared, in regard to both the development of 'off' odours and the numbers and types of bacteria present. In the second experiment only chlortetracycline was used and the effects of storing treated and untreated poultry at 5° were compared with those at 1°; no bacteriological analysis was made on these chickens.

Table 2. *The effect of treatment during cooling with tetracycline antibiotics on the development of 'off' odours during the storage of eviscerated chickens at* 1°

Treatment*	Nos. of carcases and times (days) for development of			
	Slight 'off' odour		Strong 'off' odour	
	Nos.	Time	Nos.	Time
Control	1	12	2	17
	7	13	2	18
	1	14	1	19
Means		13·0		17·8
Oxytetracycline	1	14	3	21
	1	15	2	23
	1	17	1	26
	5	19		
	1	20		
Means		17·9		22·5
Tetracycline	2	16	1	21
	2	17	3	25
	3	18	1	26
	1	20	1	29
	1	21		
Means		17·9		25·2
Chlortetracycline	1	13	1	22
	2	18	1	27
	3	20	4	30
	3	21		
Means		19·1		28·2

* There were 9 carcases for each treatment.

Effect of the antibiotics on poultry held at 1°.

Sensory evaluation of storage life. The results of the storage test on 36 chickens are shown in Table 2. All the poultry which had been treated with a tetracycline antibiotic kept longer than the control group. Statistical analysis showed that the extension of 5–6 days before a slight 'off' odour developed was highly significant. The mean storage life of 19·1 days before a slight 'off' odour developed on the poultry which had been treated with chlortetracycline was not significantly different from the means of 17·9 days for those which had been treated with oxytetracycline or tetracycline. During subsequent storage until a strong 'off' odour developed there were greater differences between the poultry treated with the different antibiotics. After the controls, the oxytetracycline treated poultry spoiled first and then those treated with tetracycline. After a mean storage time of 28·2 days, the poultry treated with chlortetracycline were considered to have a strong 'off' odour, although this was difficult to assess because its character was different from that of the other groups.

Bacteriological condition of the chickens. The chickens were sampled by method 2, and to study the changes in the spoilage flora during storage of both control and antibiotic treated poultry, colony counts were made at 1°, 20°, and 37°. It was anticipated that the 1° count would give the number of organisms which could grow during storage at 1°, that the 37° count would show faecal and other contamination, while the 20° count would include both groups of organisms. Faecal streptococci were also counted selectively as indicators of contamination from the gut, and yeasts were enumerated separately. Chickens were sampled and analysed initially, and after storage to the stages of slight 'off' odour and strong 'off' odour.

Table 3 shows the colony counts at 1°, 20° and 37° on the control chickens, initially and during storage. Agreement between the results from the several chickens sampled at each stage of spoilage was good. Initially the count at 20° was higher than those at 1° and 37°. It probably consisted of miscellaneous mesophilic organisms

Table 3. *The relationship between the colony counts* at* 1°, 20°, *and* 37° *to the development of 'off' odours in control chickens during storage at* 1°

Carcase no.	Stage of storage	Colony count† (orgs./cm²) at 1°	20°	37°
6	Initial	$1{\cdot}9\times10^{4}$	$1{\cdot}6\times10^{5}$	$3{\cdot}2\times10^{4}$
7		$4{\cdot}2\times10^{4}$	$1{\cdot}4\times10^{5}$	$1{\cdot}1\times10^{4}$
8		$4{\cdot}5\times10^{4}$	$2{\cdot}4\times10^{5}$	$8{\cdot}8\times10^{4}$
	Means	$3{\cdot}5\times10^{4}$	$1{\cdot}8\times10^{5}$	$4{\cdot}4\times10^{4}$
9	Slight	$1{\cdot}2\times10^{8}$	$1{\cdot}3\times10^{8}$	$9{\cdot}4\times10^{5}$
10	'off'	$9{\cdot}1\times10^{7}$	$8{\cdot}0\times10^{7}$	$8{\cdot}7\times10^{5}$
11	odour	$1{\cdot}4\times10^{7}$	$1{\cdot}2\times10^{7}$	$4{\cdot}4\times10^{4}$
	Means	$7{\cdot}5\times10^{7}$	$7{\cdot}4\times10^{7}$	$6{\cdot}2\times10^{5}$
12	Strong	$9{\cdot}3\times10^{8}$	$1{\cdot}3\times10^{9}$	$4{\cdot}4\times10^{6}$
13	'off'	$7{\cdot}5\times10^{8}$	$8{\cdot}5\times10^{8}$	$3{\cdot}6\times10^{7}$
14	odour	$6{\cdot}7\times10^{8}$	$9{\cdot}7\times10^{8}$	$1{\cdot}3\times10^{7}$
	Means	$7{\cdot}8\times10^{8}$	$1{\cdot}0\times10^{9}$	$1{\cdot}8\times10^{7}$

* Sampling method 2. † Each value is the mean of the counts on 2 media from 1 chicken.

which were unable to develop in the 2 week incubation period at 1° or overnight at 37°. During storage the counts at 1° and 20° became almost identical because of the multiplication of psychrophilic organisms which could also grow at 20°. The 37° count increased, but was small compared with the counts at the other temperatures. A similar relationship was found with the antibiotic treated chickens.

A comparison of the results obtained from the control chickens with those from the chickens treated with oxytetracycline and chlortetracycline, using sampling method 2, is given in Table 4, which shows the counts at 1° and the numbers of yeasts

Table 4. *The relationship between the numbers of micro-organisms* and the development of 'off' odours in stored control and antibiotic treated poultry*

Stage of storage	Type of count	Treatment, carcase no., and count of organisms (no./cm²)					
		Control		Oxytetracycline		Chlortetracycline	
		Carcase	Count	Carcase	Count	Carcase	Count
Initial	Colony count at 1°	6	$1{\cdot}9\times10^{4}$	Not tested		21	$7{\cdot}9\times10^{3}$
		7	$4{\cdot}2\times10^{4}$			22	$1{\cdot}7\times10^{3}$
		8	$4{\cdot}5\times10^{4}$			23	$2{\cdot}0\times10^{3}$
	Means		$3{\cdot}5\times10^{4}$				$3{\cdot}9\times10^{3}$
	Yeasts	6	$1{\cdot}8\times10^{3}$	Not tested		21	$1{\cdot}6\times10^{3}$
		7	$2{\cdot}5\times10^{4}$			22	$1{\cdot}4\times10^{3}$
		8	$2{\cdot}8\times10^{3}$			23	$2{\cdot}5\times10^{3}$
	Means		$9{\cdot}9\times10^{3}$				$1{\cdot}8\times10^{3}$
	Faecal strepto-cocci	6	$4{\cdot}0\times10^{3}$	Not tested		21	$2{\cdot}1\times10^{3}$
		7	$1{\cdot}0\times10^{3}$			22	$2{\cdot}6\times10^{3}$
		8	$1{\cdot}1\times10^{4}$			23	$8{\cdot}6\times10^{2}$
	Means		$5{\cdot}3\times10^{3}$				$1{\cdot}9\times10^{3}$
Slight 'off' odour	Colony count at 1°	9	$1{\cdot}2\times10^{8}$	15	$3{\cdot}3\times10^{7}$	24	$1{\cdot}3\times10^{7}$
		10	$9{\cdot}1\times10^{7}$	16	$1{\cdot}9\times10^{8}$	25	$2{\cdot}6\times10^{7}$
		11	$1{\cdot}4\times10^{7}$	17	$8{\cdot}3\times10^{7}$	26	$5{\cdot}6\times10^{6}$
	Means		$7{\cdot}5\times10^{7}$		$1{\cdot}0\times10^{8}$		$1{\cdot}5\times10^{7}$
	Yeasts	9	$4{\cdot}9\times10^{4}$	15	$2{\cdot}3\times10^{6}$	24	$2{\cdot}1\times10^{7}$
		10	$1{\cdot}0\times10^{5}$	16	$1{\cdot}9\times10^{6}$	25	$1{\cdot}7\times10^{7}$
		11	$2{\cdot}6\times10^{4}$	17	$3{\cdot}1\times10^{6}$	26	$1{\cdot}4\times10^{7}$
	Means		$5{\cdot}8\times10^{4}$		$2{\cdot}4\times10^{6}$		$1{\cdot}7\times10^{7}$
	Faecal strepto-cocci	9	$3{\cdot}8\times10^{4}$	15	$8{\cdot}0\times10^{2}$	24	$2{\cdot}2\times10^{4}$
		10	$5{\cdot}8\times10^{3}$	16	$1{\cdot}2\times10^{3}$	25	$1{\cdot}7\times10^{4}$
		11	$8{\cdot}3\times10^{3}$	17	$2{\cdot}7\times10^{2}$	26	$5{\cdot}5\times10^{2}$
	Means		$1{\cdot}7\times10^{4}$		$7{\cdot}5\times10^{2}$		$1{\cdot}3\times10^{4}$
Strong 'off' odour	Colony count at 1°	12	$9{\cdot}3\times10^{8}$	18	$4{\cdot}4\times10^{8}$	27	$8{\cdot}4\times10^{7}$
		13	$7{\cdot}5\times10^{8}$	19	$8{\cdot}2\times10^{8}$	28	$6{\cdot}4\times10^{7}$
		14	$6{\cdot}7\times10^{8}$	20	$5{\cdot}8\times10^{8}$	29	$7{\cdot}2\times10^{7}$
	Means		$7{\cdot}8\times10^{8}$		$6{\cdot}1\times10^{8}$		$7{\cdot}3\times10^{7}$
	Yeasts	12	$1{\cdot}3\times10^{4}$	18	$3{\cdot}3\times10^{6}$	27	$1{\cdot}9\times10^{7}$
		13	$1{\cdot}9\times10^{4}$	19	$7{\cdot}7\times10^{6}$	28	$7{\cdot}1\times10^{6}$
		14	$1{\cdot}3\times10^{4}$	20	$9{\cdot}1\times10^{5}$	29	$2{\cdot}1\times10^{7}$
	Means		$1{\cdot}5\times10^{4}$		$4{\cdot}0\times10^{6}$		$1{\cdot}6\times10^{7}$
	Faecal strepto-cocci	12	$2{\cdot}2\times10^{3}$	18	$2{\cdot}1\times10^{4}$	27	$6{\cdot}2\times10^{3}$
		13	$1{\cdot}8\times10^{4}$	19	$5{\cdot}8\times10^{4}$	28	$2{\cdot}5\times10^{4}$
		14	$1{\cdot}3\times10^{5}$	20	$1{\cdot}1\times10^{5}$	29	$8{\cdot}7\times10^{4}$
	Means		$5{\cdot}0\times10^{4}$		$6{\cdot}3\times10^{4}$		$3{\cdot}9\times10^{4}$

* Sampling method 2.

and faecal streptococci. There was close agreement between the stage of 'off' odour which was developed and the numbers of micro-organisms present, particularly when account is taken of the considerably greater metabolic activity per cell of yeasts compared with bacteria (see below).

A detailed analysis was made of the psychrophilic spoilage floras to discover whether there were any differences between the types of organisms causing spoilage of the control and the antibiotic treated poultry, and the results are shown in Fig. 2. In both control and chlortetracycline treated chickens the initial populations were similar in their proportions of members of the genera *Pseudomonas* and *Achromobacter*. Included in the small group of miscellaneous bacteria were representatives of *Cytophaga* and *Flavobacterium*, as well as some fermentative organisms related to the coli-aerogenes group. On both the control chickens and those treated with oxytetracycline the predominant spoilage organisms were non-pigmented and pigmented pseudomonads, the former considerably outnumbering the latter, but where chlortetracycline had been used the *Pseudomonas* strains were completely restrained, the main spoilage organisms at slight 'off' odour being yeasts and strains of *Achromobacter*. This difference probably accounted for the 'off' odour being quite different from that of either the controls or the oxytetracycline treated poultry. Pigmented pseudomonads were present on the chlortetracycline treated carcases when a strong 'off' odour had developed, but they were outnumbered by the achromobacters and yeasts which together comprised 70% of the population.

The *Achromobacter* strains isolated from the stored chickens treated with oxytetracycline or chlortetracycline were different from those found initially or on the control

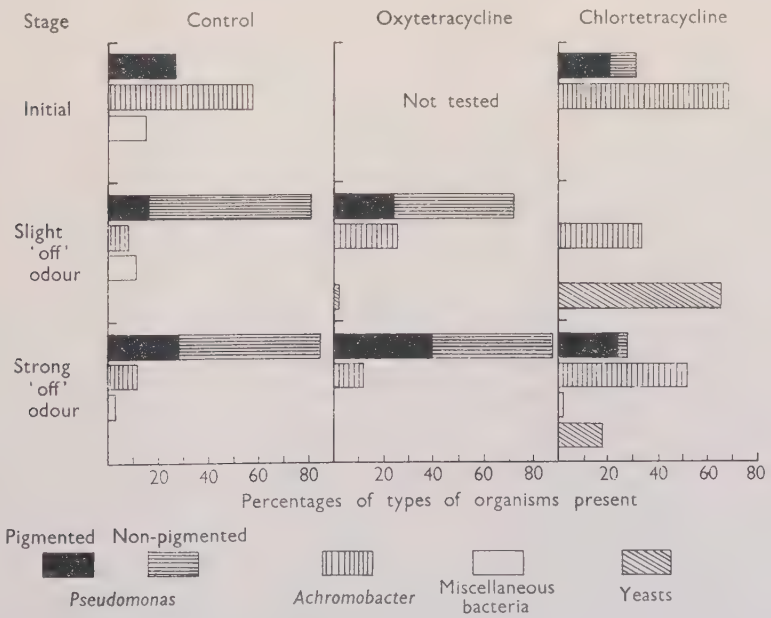

Fig. 2. The changes in the types of psychrophilic spoilage organisms on control and antibiotic treated chickens during storage.

chickens, in that they were resistant to the tetracycline compounds. Only one such strain was found on chickens before antibiotic treatment. A summary of the sensitivity to oxytetracycline of the strains of *Pseudmonas* and *Achromobacter* which were isolated is given in Table 5. All the *Achromobacter* strains were non-motile, except one isolated from an initial sample and those isolated from the control birds after storage to strong 'off' odour.

Table 5. *The sensitivity to* 10 *p/m of oxytetracycline of the strains of* Achromobacter *and* Pseudomonas *isolated*

Condition of carcases	*Achromobacter*			*Pseudomonas*					
				Pigmented			Non-pigmented		
	No. of strains	Sensitivity†		No. of strains	Sensitivity†		No. of strains	Sensitivity†	
		+	−		+	−		+	−
Controls: initial and stored	41	40	1	35	14	21	47	41	6
Chlortetracycline treated, initial	12	12	0	4	0	4	2	2	0
Chlortetracycline or oxytetracycline treated, stored	40	5	35	18	2	16	26	21	5

† + sensitive; −, resistant.

The faecal streptococci were as numerous at the slight and strong 'off' odour stages on the chlortetracycline treated poultry as on the controls (Table 4). However, these birds had received a supplement of 5 p/m of chlortetracycline throughout their life and Barnes (1958) has already shown that faecal streptococci from the intestines of birds fed on diets containing chlortetracycline supplements have a high degree of resistance to that antibiotic. As initial samples were not taken from the oxytetracycline treated poultry, no inference can be drawn from the relatively small numbers of faecal streptococci present at the slight 'off' odour stage. When the strong 'off' odour stage had been reached the numbers equalled those on the chlortetracycline treated poultry.

Distribution of micro-organisms within the visceral cavity. It was thought that there might be an uneven distribution of micro-organisms within the visceral cavity, due possibly to heavier contamination around the vent or, alternatively, to an uneven distribution of the antibiotic which enters the cavity in the ice slush used for cooling. Various regions within the visceral cavity and in the deep muscle tissue (see Fig. 1) were selected for analysis, the numbers of micro-organisms being determined by method 3. Twenty-two of the stored control and antibiotic treated chickens were examined in this way. In general, the distribution of micro-organisms was uniform throughout the cavities irrespective of the treatment received, but where there was variation the greatest numbers were close to the vent (see Fig. 1, regions 5, 8, 10, 12, 14, 16). At all stages of storage Gram-negative bacteria and/or yeasts were dominant. This finding is in agreement with the results of the colony counts reported above.

Because there was a similar distribution of micro-organisms in both treated and

untreated chickens, only the results obtained with those treated with tetracycline are shown in Table 6. Apart from the kidneys (regions 3–8) the variation in numbers of bacteria throughout the cavity was not greater than eighteenfold and in 4 of the 6 chickens the variation was less than six-fold. In this treatment yeasts constituted only a minor part of the flora. Small numbers of micro-organisms were detected in samples of the pectoral muscle of chickens with a slight 'off' odour: these were possibly there because of the nature of the sampling technique, and represented the extent of the 'carry over' of micro-organisms from contamination on the surface. In chickens 33 and 35, which had a strong 'off' odour, the numbers of bacteria in the pectoral muscle were much greater, and although their presence might have been due to the method of sampling, no comparable numbers were observed in the pectoral muscle of any other chicken.

Table 6. *The distribution of micro-organisms within the visceral cavities and pectoral muscles of tetracycline treated poultry during storage at* 1°

Carcase: Condition	No.		Micro-organisms present† (10^6/cm^2) in: Visceral cavity regions: 1, 2	3–8	10, 12	14, 16	13, 15	Pectoral muscle regions 17–20
Slight 'off' odour	30	Bacteria*	58	18	68	240	—	<1·4
		Yeasts	<0·2	<0·2	1·4	0·6	—	0·6
	31	Bacteria*	320	<2·2	62	55	250	8·8
		Yeasts	0·2	0·9	1·3	1·1	1·1	<0·3
	32	Bacteria*	8·6	<2·2	2·2	—	5·5	<2·2
		Yeasts	0·4	1·0	0·7	—	0·9	<0·2
Strong 'off' odour	33	Bacteria*	90	<22	540	300	—	23
		Yeasts	1·4	2·0	1·3	0·4	—	<0·2
	34	Bacteria*	75	250	670	910	—	11
		Yeasts	<0·2	0·7	32	—	—	<0·2
	35	Bacteria*	66	<2·2	1200	480	—	150
		Yeasts	<0·7	1·5	<2·3	1·0	—	<0·2

* Gram-negative organisms only; the numbers of Gram-positive organisms (including cocci) were small. † Sampling method 3.

Table 7. *Changes in the microbial population in the visceral muscle, regions* 14 *and* 16 (*Fig.* 1), *of control and antibiotic treated poultry during storage at* 1°

Treatment	Nos. of micro-organisms* (10^6/cm^2) after storage to: Slight 'off' odour: Gram −ve bacteria	Yeasts	Strong 'off' odour: Gram −ve bacteria	Yeasts
Control	33	<1	670	<1
Chlortetracycline	<11	4·3	<2·2	57·7
Tetracycline	98	0·6	500	0·7
Oxytetracycline	5·4	0·8	Not tested	

* Each value is the mean of the counts for 3 carcases, with sampling method 3.

The relationship between the numbers of Gram-negative bacteria and yeasts in regions 14 and 16 (Fig. 1) and the development of slight and strong 'off' odours in control and antibiotic treated chickens, is shown in Table 7. These regions were selected because they represent the most heavily contaminated areas of the visceral cavity. The relative numbers of bacteria and yeasts agree closely with those obtained (Table 4) when sampling method 2 was used.

Changes in the pH of the visceral muscle during storage. The changes in pH in regions 10 and 12 (Fig. 1) during storage were followed, with simultaneous estimations of the numbers of micro-organisms present, and an attempt was made to correlate these observations. However, it is impossible to equate chemical activity to total counts when these comprise both bacteria and yeasts, because the rates of metabolic turnover of these organisms and yeasts differ by the order of 10^3 on a cell number basis. Therefore, numbers of organisms have been converted to cell metabolic rates, Q_{O_2} (cell), using data from Spector (1956) for *Sarcina lutea*, Q_{O_2} (cell)=0·007 μl. O_2/cell/hr, and *Saccharomyces* spp., Q_{O_2} (cell)=1·8 μl. O_2/cell/hr, to represent the bacterial and yeast floras respectively. These values are necessarily approximate, not only because of the considerable variations in the sizes of the cells of both bacteria and yeasts, but also because budding yeast cells were counted as single units. Nevertheless, the difference in size between the two types of organism is so large that the orders of their respective rates of metabolic turnover can be calculated from this information.

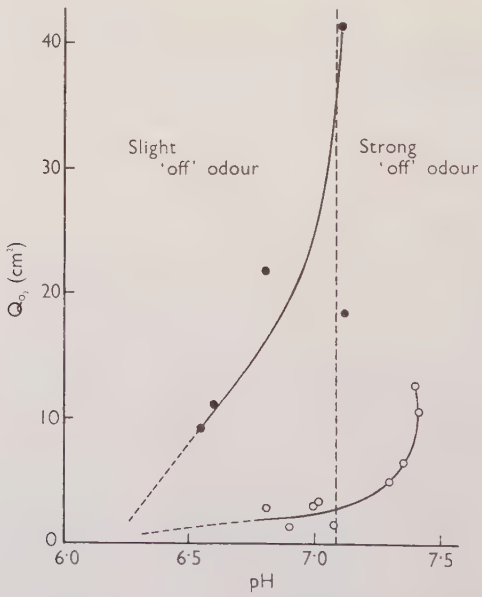

Fig. 3. The relationship between the pH of the visceral muscles and the rates of metabolic turnover of the spoilage floras.
Ordinate: Q_{O_2}(cm²) represents the no. of microlitres of O_2 taken up in 1 hr by the microflora on 1 cm² of muscle surface, and is equal to Q_{O_2} (cell) × no. of organisms/cm².
Full circles, yeasts predominant. Open circles, bacteria predominant.

The relationship between the derived metabolic rates and the pH values is shown in Fig. 3. All chickens with a strong 'off' odour, irrespective of their treatment during cooling, had a visceral muscle pH of 7·1 or higher, but where yeasts accounted for more than 97% of the metabolic turnover the pH did not rise above 7·1, in spite of the high rate of metabolism compared with that in similar areas of muscle surface where bacteria were the dominant organisms. In the latter case the pH of the muscle rose to 7·4.

Fluorescence within the visceral cavities during storage. It has been suggested by Cotterill (1956) that the presence or absence of fluorescence in a stored carcase can be used as an indication of its bacteriological condition, possibly on the assumption that the organisms usually producing spoilage at low temperatures are pigmented pseudomonads. In this experiment all the chickens which were dissected were also examined under ultraviolet light. A yellow-green fluorescence was sometimes observed on the skin of chickens treated with chlortetracycline and for about a week after storage, but it was never observed within the visceral cavities of the stored chickens. The incidence of fluorescence in the different regions is shown in Table 8. It occurred irregularly in chickens with a slight 'off' odour, none being observed in 5 of the 12 carcases examined. The only group in which fluorescence was a consistent feature was that treated with tetracycline; in contrast, the group treated with chlortetracycline showed no fluorescence. When a strong 'off' odour had developed all the chickens showed some fluorescence, but it was again most widespread in the group which had been treated with tetracycline.

Table 8. *The occurrence of fluorescence within the visceral cavities of control and antibiotic treated poultry stored at* 1°

Treatment	Presence (+) or absence (−) of fluorescence at the stage of											
	Slight 'off' odour						Strong 'off' odour					
	Carcase no.	Visceral cavity				Pectoral muscle	Carcase no.	Visceral cavity				Pectoral muscle
		1, 2	3–8	10, 12	14, 16	13, 15	17–20		1, 2	3–8	10, 12	14, 16	13, 15	17–20
Control	10	−	−	−	−	−	−	12	−	+	+	+	+	−
	11	−	−	−	−	−	−	13	−	+	+	+	+	−
	36	−	−	+	−	+	−	14	−	−	+	−	+	−
Chlortetracycline	24	−	−	−	−	−	−	27	−	−	−	−	+	−
	25	−	−	−	−	−	−	28	+	−	+	−	+	−
	26	−	−	−	−	−	−	29	−	−	−	+	−	−
Tetracycline	30	−	+	+	+	+	−	33	−	−	+	+	−	−
	31	+	+	−	+	+	−	34	+	−	+	+	+	−
	32	+	−	−	−	−	−	35	−	−	+	+	+	−
Oxytetracycline	15	−	+	−	−	−	−	Not tested						
	16	−	−	−	−	+	−							
	17	−	+	−	−	−	−							

Comparison of 1° *and* 5° *for the storage of control and chlortetracycline treated poultry.* The times of appearance of 'off' odours among control and chlortetracycline treated poultry stored at 1° and 5° are shown in Table 9. Lowering the temperature from

5° to 1° extended the storage life from 9 days to 13 days. Treatment with chlortetracycline and storage at 5° gave a 4 day extension of storage life, so that holding control chickens at 1° or chlortetracycline treated chickens at 5° was equally effective.

Table 9. *The effect of temperature and chlortetracycline treatment on the rate of development of 'off' odours in stored eviscerated chickens*

Antibiotic treatment	Storage temp. (°C)	Nos. of carcases	Slight 'off' odour: Time (days)	Strong 'off' odour: No.	Strong 'off' odour: Time (days)
Control	5	3	8	5	11
		4	9	3	12
		3	10	2	13
	Means		9·0		11·7
	1	1	12	2	17
		7	13	2	18
		1	14	1	19
	Means		13·0		17·8
Chlortetracycline	5	1	11	5	16
		1	12	4	17
		7	13	1	18
		1	14		
	Means		12·8		16·6
	1	1	13	1	22
		2	18	1	27
		3	20	4	30
		3	21		
	Means		19·1		28·2

Discussion

Each of the tetracycline antibiotics was similarly effective as a preservative for eviscerated poultry which had been frozen, transported without refrigeration and then held at 1°. Kohler *et al.* (1956–57) had previously found that chlortetracycline was effective under similar conditions. It is significant, as few refrigerators in retail shops or households are kept at temperatures as low as 1°, that lowering the holding temperature from 5° to 1° was as effective in extending the storage life of eviscerated poultry as using a tetracycline antibiotic, in this instance chlortetracycline. Previously Shrimpton, Barnes & Miller (1958) emphasized, in the case of uneviscerated poultry, that storage at low temperatures is a safer way of controlling spoilage than the use of antibiotics.

When numbers of micro-organisms were determined by the somewhat unusual method of combining a sample from the skin of the chicken with one from the visceral cavity, there was no discrepancy between the first detection of 'off' odours and the total numbers of organisms present. The finding of 10^7–10^8 organisms/cm^2 of chicken agrees well with the results given by Ayres *et al.* (1956) for antibiotic treated poultry and by Haines & Smith (1933) for the slime level on meat carcases. Similar results were also obtained by Thornley (1958, pers. comm.) with irradiated poultry, using

the same method. Evidently those two sites were among the most heavily contaminated areas, and this was demonstrated for the visceral cavity by the direct microscopical counts carried out on other areas on the same carcases.

Comparison of the data in Tables 1 and 4 makes it evident that the numbers of psychrophiles on the control chickens increased tenfold during the cooling in ice slush and the subsequent trussing and packaging prior to freezing, while there was no increase on the chlortetracycline treated chickens. But Haines & Smith (1933) showed that when meat was held at 0° a tenfold decrease in the initial numbers of spoilage organisms gave approximately 3 days extra storage life. Thus almost 50% of the additional storage life of the antibiotic treated poultry was probably due to the prevention by the antibiotic of multiplication of the psychrophilic bacteria on the eviscerated washed chickens during chilling and between chilling and freezing.

The proportions of representatives of the genera *Pseudomonas* and *Achromobacter* present at the start of storage were similar in the control and chlortetracycline treated chickens, but during storage to the stage of slight 'off' odour the pseudomonads were completely suppressed by that antibiotic, spoilage being due to achromobacters and to yeasts. In the spoiled control and oxytetracycline treated chickens the actively motile *Pseudomonas* strains considerably outnumbered the non-motile *Achromobacter* strains. Although initially the pigmented *Pseudomonas* strains predominated, spoilage was due mainly to non-pigmented strains. From the few observations made of colony size and rate of growth in broth at 1° there were no obvious differences between their growth rates at that temperature, but mean generation times were not determined.

The growth of non-pigmented pseudomonads during storage (see Fig. 2) explains the poor correlation between the development of 'off' odours and the appearance of fluorescence (Table 8). Fluorescence only indicates spoilage by pigmented strains of *Pseudomonas* and a negative finding does not necessarily mean a satisfactory bacteriological condition. With chickens treated with tetracycline there is evidence from the fluorescence results that pigmented pseudomonads were present in greater numbers than in the other groups of chickens. However, no analysis was made of the psychrophilic population.

Different spoilage floras developed in the presence of the three tetracyclines and this came about because several inter-related factors were acting. First, although the three tetracyclines are active against the same groups of organisms, chlortetracycline is about five times as potent as either oxytetracycline or tetracycline, and this difference may well be emphasized by the fact that the concentration of antibiotic in the poultry after dipping for 2 hr in ice slush containing 10 p/m is always less than 7 and usually less than 3 p/m. Second, Shewan *et al.* (1954) showed that while the non-pigmented strains of *Pseudomonas* were inhibited by 10 p/m of oxytetracycline, most of the pigmented strains were more resistant (Table 5). Thus it is reasonable to suppose that while the chlortetracycline completely inhibited both kinds of pseudomonads during the early part of the storage period, the oxytetracycline was less effective. Comparison with the results for the control chickens shows that there was a higher proportion of the pigmented strains in the oxytetracycline treated poultry. The pigmented pseudomonads which developed during

further storage of the chlortetracycline treated chickens to the strong 'off' odour stage may have grown because of a gradual decrease in the concentration of the antibiotic.

The *Achromobacter* strains which developed on the antibiotic treated chickens were resistant to 10 p/m of oxytetracycline, although still sensitive to penicillin. Only one such strain was found anywhere on poultry which had not been treated with an antibiotic, and this was on a carcase prior to freezing. More than one-third of the strains of *Achromobacter* isolated from the stored, antibiotic treated poultry formed very gelatinous colonies and were probably not easily removable by the rinsing technique. Anomalies in the relationship between 'off' odours and numbers of organisms would then occur. The pseudomonads are responsible for the characteristic odour of chilled spoiled poultry, and when these are replaced by *Achromobacter* strains and yeasts, as in chlortetracycline treated chickens, the odour is completely changed.

The pH of the visceral muscle rose both when it was spoiled by a predominantly pseudomonad flora and when an *Achromobacter*-yeast flora was responsible. Also, a pH of 7·1 was associated with the first development of a strong 'off' odour in both types of spoilage (Fig. 3). There is, therefore, some similarity in the chemical changes in flesh spoiling from both types of flora. However, pH cannot be used as an indicator of the numbers of micro-organisms on the muscles of the visceral cavity without detailed information of the *post-mortem* physiological changes in those muscles, which may also influence their pH.

In these experiments the faecal streptococci were used as indicators of faecal contamination, and the results showed that chlortetracycline had no inhibitory effect on the faecal flora of birds fed on a chlortetracycline supplemented ration. This fact emphasizes the importance of keeping eviscerated poultry at low temperatures because at 1°, for example, any resistant pathogens which might be present would be unable to multiply.

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THE SOCIETY FOR APPLIED BACTERIOLOGY

Autumn Meeting, 1957

An Autumn Meeting, held in the afternoon of Tuesday, 22 October, 1957, took the form of a visit to the Laboratories of the Metropolitan Water Board, by kind permission of the Director.

Winter Meeting

The Winter Meeting was held in the Barnes Hall of The Royal Society of Medicine, 1, Wimpole Street, London, W.1, on Wednesday, 8 January, 1958. In the morning there was a short business meeting, followed by a paper reading session. In the afternoon there was a Joint Meeting with the Microbiology Group of the Society of Chemical Industry, when the subject under discussion was 'New Techniques in Microscopy'.

Morning Session. The following papers were read:—

The Use of the Membrane Filter in the Estimation of Thermophilic Spores in the Preparation of Canners' Sugar. By Sheila J. Attenborough and M. Pamela Scarr (*Tate & Lyle, Ltd., Research Laboratories, Keston, Kent*). (See *J. appl. Bact.* **20**, 460).

Observations on the Microflora of Whole Eviscerated Chicken Irradiated with Cobalt 60 Gamma Rays. By Margaret J. Thornley (*Low Temperature Research Station, Downing Street, Cambridge*).

Chicken carcases, eviscerated by two different methods, were loosely packed in polythene bags and irradiated with Cobalt 60 gamma rays. Doses from 125,000–825,000 rads were used, and a 10 p/m chlortetracycline (CTC) dip was also used, alone and combined with 250,000 rads. Storage at 1–3° was terminated when 'off' odours became marked; these were sometimes of an unusual type in irradiated birds. Storage life was extended 2–3½ times by a dose of 500,000 rads, 2½ times by 250,000 rads with CTC, and 6 times by 825,000 rads, without significant change in the flavour of the cooked birds immediately after irradiation.

Among the few bacteria surviving irradiation, the proportion of Gram-positive types was greater than on the controls, and with the higher doses yeasts predominated. Nevertheless, yeasts were only important in the eventual spoilage flora where 250,000 rads was combined with CTC; in all other cases, Gram-negative bacteria were most numerous. On the controls, these were mainly non-pigmented pseudomonads, and on irradiated birds, achromobacters in 2 of the 3 experiments. In the 3rd experiment, pigmented and non-pigmented representatives of *Pseudomonas* were most numerous on the irradiated birds.

Thus, although changes due to irradiation were observed in the low temperature spoilage flora of chicken carcases, together with unusual odours on spoilage, only organisms normally present on chilled meat were involved.

Observations on the Drop Method for Colony Counts. By J. G. Davis and Jean S. Bell (*Dr. J. G. Davis & Partners, 9, Gerrard Street, London, W.1*). (See *J. appl. Bact.* **21**, 217).

The Fate of *Staphylococcus aureus* in Cheddar Cheese. By A. T. R. Mattick, F. K. Neave, Helen R. Chapman and N. J. Berridge (*National Institute for Research in Dairying, Shinfield, Reading, Berks*).

Milk from *Staphylococcus aureus* infected cows was pasteurized, two identifiable strains of *Staph. aureus* added and four Cheddar cheese made from the milk. The fate of the two strains was as follows: there was an 8- to 21-fold increase during the making of the cheese, followed by a fairly rapid decline. The decline was least rapid in the sweet cheese. *Staph. aureus* was not recovered after 16-22 weeks storage at 59°F (15°). The cheese, at six months, appeared normal and were well liked. No ill effects were caused by its consumption.

Afternoon Session. The following papers were read at the Joint Meeting with the Microbiology Group of the Society of Chemical Industry, on the subject of

New Techniques in Microscopy

Recent Advances in Electron Microscopical Techniques. By V. E. Cosslett (*Cavendish Laboratory, Cambridge*). (See *J. appl. Bact.* **21**, 128).

Ultraviolet Microscopy. By J. Smiles (*National Institute for Medical Research, Mill Hill, London, N.W.7*). (See *J. appl. Bact.* **21**, 137).

The Use of Phase Contrast Microscopy in Microbiology. By W. H. Hughes (*The Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2*). (See *J. appl. Bact.* **21**, 143).

The Measurement of Mass and Concentration in Micro-Organisms. By R. Barer (*Department of Human Anatomy, University of Oxford*). (See *J. appl. Bact.* **21**, 146).

Dr. Hughes' paper was illustrated by a short film.

Annual General Meeting and Conference

The 28th Annual General Meeting and Conference was held in Wills Hall, University of Bristol, on 8, 9 and 10 July, 1958. Members assembled on Monday, 7 July, and on the morning of Tuesday, 8 July, there was a paper reading session. In the afternoon a visit was paid to the Research Station, Long Ashton, University of Bristol, by kind permission of the Director, Professor H. G. H. Kearns. On Wednesday, 9 July, the Annual General Meeting was held, and the rest of the day was devoted to paper reading. The Annual Society Dinner was held in the evening, in Wills Hall. On the morning of Thursday, 10 July, there was another paper reading session, and in the afternoon there was an excursion to Cheddar Gorge and Wells.

The following papers were read on 8 July:—

Microbiological Aspects of Fruit Juice Processing. By Marion Allan and H. Fore (*Beecham Foods Ltd. (Carters of Coleford), The Royal Forest Factory, Coleford, Glos.*).

The investigations on the process used for the production of blackcurrant juice from whole fruit were confined to the yeasts and moulds. Three yeasts (as yet unidentified) and seventeen species of moulds were isolated during the 1957 fresh fruit season.

Overall counts of yeasts and moulds at various stages in the process between harvest and juice storage were obtained in the 1956 and 1957 fresh fruit seasons. The general picture showed an increase in count on the whole fruit between picking and processing. Little change occurred, however, during the early stages of processing and the absence of an increase in the count could possibly be accounted for by mycelial yeasts on the whole fruit failing to survive in the changed environment.

Comparatively low counts were obtained on pressed juice and these were reduced by centrifugation and, more markedly, by filtration. Delay between the various stages of the process resulted in build-up of yeast count but the final stage of flash-pasteurization produced a juice suitable for storage at 1–2° under 45 lb/in.^{2} of carbon dioxide.

There was evidence of a general increase in counts as the fresh blackcurrant season progressed.

Yeast Flora of Apple Juices and Ciders. By F. W. Beech (*Research Station, Long Ashton, Bristol*). (See *J. appl. Bact.* **21**, 257.)

Lactic Acid Bacteria as Spoilage Organisms of Fruit Juice Products. By J. G. Carr (*Research Station, Long Ashton, Bristol*). (See *J. appl. Bact.* **21**, 267).

Some Effects of Trace Metals on Hydrogenase Activity and Nitrogen Fixation in *Azotobacter* spp. and in *Clostridium pasteurianum*. By D. J. D. Nicholas (*A.R.C. Unit of Plant Nutrition, Research Station, Long Ashton, Bristol*).

Although iron and molybdenum are essential for nitrogen fixation in *Azotobacter* spp., the iron requirement is often about fifty times that for molybdenum, e.g. in *A. chroococcum* the amounts (in μg/ml of culture solution) required for optimum fixation are: Mo, 0·01; Fe, 0·5. All the species so far examined have a wider tolerance for molybdenum than for iron since the former does not become toxic until 0·1 mg/ml of culture solution is reached. In *A. chroococcum*, the molybdenum requirement can be partially satisfied (to the extent of 70%) by substituting sodium vanadate for sodium molybdate, but in other strains there is no such replacement.

Hydrogenase, the enzyme that oxidizes molecular hydrogen, is markedly reduced in iron-deficient cells of both *A. vinelandii* and *Clostridium pasteurianum*. A deficiency of molybdenum, however, does not reduce the enzyme in *Azotobacter* and has little effect on the one in *Clostridium*. Since these results are independent of cell yields it is clear that iron is primarily required for hydrogenase activity whereas both metals are essential for nitrogen fixation. Excessive amounts of sodium tungstate, a well-known antagonist of molybdate, markedly depressed nitrogen fixation but were without effect on hydrogenase activity.

The hydrogenase enzyme is best extracted by crushing cells of either organism in a Hughes press at −20° and suspending the material in 0·2 M tris-(hydroxymethyl)-aminomethane buffer (pH 8·0) containing 10^{-2} M sodium dithionite; the latter is essential to keep the enzyme reduced, since it is readily inactivated in air. The enzyme from *Azotobacter* or *Clostridium* will reduce a range of acceptors including methylene blue, potassium ferricyanide, diphosphopyridine nucleotide and cytochrome *c*.

The following papers were read on 9 July:—

Comparisons of Different Media for Counting Sugar-Tolerant Yeasts in Concentrated Orange Juice. By M. Ingram (*Low Temperature Research Station, Downing Street, Cambridge*).

To select and count the sugar tolerant yeasts which ferment sixfold concentrated orange juice, an agar medium was developed containing 50% glucose plus 1% citric acid and 1% tryptone; it is incubated for 4–5 days at 25°.

The medium has disadvantages: it is troublesome to prepare, and the colonies grow slowly and are translucent. These properties result directly from the high sugar concentration, on which the selective action of the medium depends.

Counts on this medium were compared with those on potato dextrose or nutrient dextrose agars. Usually they were virtually the same. Where the latter were the larger, they indicated contamination by alien yeasts incapable of fermenting concentrate.

Potato dextrose was preferable to nutrient dextrose agar, as the latter recorded

irrelevant bacteria and occasionally failed to record small numbers of the sugar tolerant yeasts.

It is suggested that the above would also be applicable to other sugar rich concentrates of not less than 50° Brix.

Storage Rots of Citrus Fruits. By N. J. Butler (*Galloway & Barton-Wright, London*).

The identification of the organisms producing storage rots and the elucidation of the sequence of organisms when more than one is involved may yield much information regarding the past history of the fruit and the factors that contributed towards rot development. The commonest rots are: *Penicillium* rots (*P. digitatum* and *P. italicum*); stem end rots (e.g. *Phomopsis* and *Diplodia*), *Alternaria* rots (*A. citri*); and sour rot (*Oospora citri-aurantii*).

The conditions which favour the development of the commoner rots are: fruit of low vitality (e.g. stem end types, *Alternaria* and *Botrytis*); mechanical injury (*Penicillium*, *Colletotrichum* and *Oospora* in part); mature fruit (*Oospora*, *Penicillium* and *Phomopsis*); and long storage (*Oospora*, stem end types, and *Alternaria*).

Control measures during harvesting, transport and storage include minimizing injury, antiseptic washes, fumigation, antiseptic impregnated wrappers, and observance of safe storage times and temperature. During marketing rapid distribution is aimed at and when rots have developed which spread by contact it is necessary to repack and discard the affected fruit. Types which spread by contact are *Penicillium italicum*, *Oospora*, *Sclerotinia* and *Phytophthora*.

Microbiological Aspects of Banana Transport. By N. J. Butler (*Galloway & Barton-Wright, London*).

Bananas are transported green and for long journeys are carried in refrigerated tankers. The usual temperature during transport is 53–54°F and ripening is carried out at temperatures in the range 60–70°F. The rots developing on bananas during transport and ripening may be classed as stalk rots, stem rots and fruit rots. If stalk rot is severe the affected portion may collapse and this may lead to a partial collapse of the cargo. Severe stem rots result in 'finger dropping'. The organisms producing wastage may be grouped as those capable of development on green fruit; those which only develop on ripening fruit; those which develop well at 53°F; and those which develop on the fruit before cooling.

Many organisms produce wastage but only two are of serious consequence on bananas imported into Great Britain and Europe, viz. *Thielaviopsis paradoxa* and *Gloeosporium musarum*. Both these organisms are wound parasites. *T. paradoxa* will develop on green fruit, grow rapidly at 53°F, and produces stalk rot, stem rot and finger dropping, and fruit rot. *G. musarum* only develops on ripening fruit as it requires sugar for development, is slow developing at 53°F, and produces stem rot with finger dropping and anthracnose scarring of the fruit.

Control measures comprise reducing injury by careful handling and packing stalks in individual polythene bags; sealing fresh cut stalk ends to control stalk rot; and rapid cooling to 53°F and rapid delivery from the plantation to the ship to avoid the development of high temperature organisms.

A Study of a Gram-positive Coccus Responsible for Ropiness and Viscosity in Malt Wort. By J. W. Hopton and A. N. Hall (*Department of Industrial Biochemistry, The Manchester College of Science and Technology, Manchester*, 1).

A Gram-positive coccus which possesses properties typical of the pediococci produces a ropy, viscous growth in malt wort. Nutritional studies, using the synthetic medium of Dunn *et al.* (1947), showed that, of eight vitamins investigated, only pantothenic acid was required by the organism but growth did not occur in the complete medium unless a trace of malt wort was added. In a medium consisting of mineral salts, glucose and alcoholic extracts of peptone and yeast autolysate the organism grew well and the viscosity of the medium increased. Higher viscosities were attained if the buffering capacity of the medium was raised either by increasing the concentration of the alcoholic extracts or by incorporating acetate. The material responsible for viscosity was produced in only small amounts and appeared to be of the nature of a mucopolysaccharide. The only sugar obtained by acid hydrolysis of this material was glucose. Fructose, maltose, sucrose and raffinose were also fermented but only in medium containing maltose was the increase in viscosity comparable with that in medium containing glucose.

Reference

Dunn, M. S., Shankman, S., Camien, M. N. & Block, H. (1947). The amino acid requirements of twenty three lactic acid bacteria. *J. biol. Chem.* **168**, 1.

Calcium Requirements of *Azotobacter*. By J. R. Norris and H. L. Jensen (*State Laboratory for Soil and Crop Research, Lyngby, Denmark*).

Although various workers have studied the calcium requirements of *Azotobacter*, there is still considerable disagreement on several points, especially over the question of the dispensability of calcium when growth occurs in the presence of combined nitrogen.

Calcium was shown to be essential for the growth of *A. vinelandii*, *A. beijerinckii*, *A. chroococcum* and *A. insigne* in the presence and absence of combined nitrogen. *A. agile* and *A. macrocytogenes* were able to grow in the absence of calcium but growth was stimulated by the cation. A strain of *A. agile* which grew poorly in the absence of calcium could be adapted to grow steadily in its absence by passage in calcium-free medium. Calcium could be replaced by strontium at roughly the same molar concentrations.

The authors present evidence suggesting that the calcium-sparing effect of ammonium and sodium acetates reported in the literature may be dependent on the size of inoculum used. Using minimal inocula such sparing effects could not be demonstrated.

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The Reduction of the Biochemical Oxygen Demand (B.O.D.) of Whey, by Controlled Mould Growth. By D. J. Cook and M. Woodbine (*Microbiological Unit, Department of Agricultural Sciences, University of Nottingham*).

The ability of fat producing moulds to utilize the lactose in whey has been surveyed and the effect of low levels of nitrogen supplements on the growth of moulds on whey studied. As a corollary to this work, experiments involving the supplementation of whey with various mineral salts have now been done. The aim of these experiments was to increase the growth on whey of various species of moulds, particularly *Penicillium frequentans*.

From screening experiments, it was found that relatively high levels of potassium sulphate and ammonium nitrate supplements substantially increased the growth of *Penicillium frequentans* on whey and that a mixture of 0·2 M potassium sulphate and 0·1 M ammonium nitrate was particularly effective. Fat production was also substantially increased by this supplement. Further, the increase in the production of fat (%) by the potassium sulphate and ammonium nitrate supplement was greater than the corresponding increase in felt weight (%). Other experiments have also pointed to the possibility of selective stimulation of mould fat production by potassium sulphate supplementation.

The B.O.D. of unsupplemented whey, estimated by the 'alkaline azide' modification of the Winkler method, first suggested by Kessener (1933), was found to vary between 3,000 and 6,000 parts per 100,000. The mixture of 0·2 M potassium sulphate and 0·1 M ammonium nitrate increased this by approximately 500 parts per 100,000. However, the increase in the growth rate induced by the supplement would seem to offset any effect of the supplement in increasing the B.O.D. of whey.

It was found that the reduction in the B.O.D. closely followed the utilization of whey lactose by *Penicillium frequentans* in the initial stages of the growth period. The reduction of the B.O.D. of whey was not checked during the growth period and, towards the end of the 40 day growth period, when the rate of lactose utilization tended to fall off, the rate of the reduction in B.O.D. was maintained. After 40 days growth the B.O.D. of whey was reduced to between 1 and 2% of its original value, i.e. for the sample of whey used in this experiment from 6,000 to 60 parts per 100,000 for unsupplemented whey and from 6,500 to 114 parts per 100,000 for supplemented whey.

Reference

Kessener (1933). *Annu. Conf. Publ. Wks, Roads and Transport Services*, no. 24.

The Microbiological Examination of Butter. By R. G. Druce and S. B. Thomas (*National Agricultural Advisory Service, Trawscoed, Aberystwyth*).

Butter made on farms and at creameries was examined microbiologically within 2 hr of production. Various faults in manufacturing practice such as contaminated water supplies, unsterile utensils, post-pasteurization contamination and inadequate refrigeration facilities were reflected in an increased microbial content of the butter as shown by the various tests employed.

Colony counts of psychrophiles (Yeastrel milk agar, 14 days at 3–5°), coli-aerogenes organisms (Violet red bile agar, 24 hr at 30°), and yeasts and moulds (on malt agar, 5 days at 22°) were the most useful and economical tests for providing information on hygiene in manufacture. The fact that coli-aerogenes contamination could be detected within 24 hr proved extremely valuable. The following satisfactory levels are recommended for creamery butter: psychrophiles, $<10^3$/g; coli-aerogenes organisms, <10/g; yeasts and moulds, <50/g.

These three determinations are simple and easily carried out in creamery laboratories equipped for normal bacteriological control work. When combined with a keeping quality test at 22° on the butter sample they provide information which should enable the maker to control and improve the keeping quality of the butter.

The Bacterial Content of Farm Dairy Equipment. By D. G. Griffiths and S. B. Thomas (*National Milk Testing Service and National Agricultural Advisory Service, Trawscoed, Aberystwyth*).

The results obtained during the examination of 2,310 advisory rinses of farm dairy equipment by means of colony count on Yeastrel milk agar at 30°, coli-aerogenes test at 30° and milk spoilage organisms test at 22° were discussed, in relation to the attainment of proposed satisfactory colony count levels of less than 10^4 colonies/ft.^{2}

Much better results were obtained with rinses of metal equipment than with rinses of rubber parts. Only 22% of the rinses of milking machine clusters attained the satisfactory level proposed, compared with 36% in the case of metal parts. As many as 40% of the clusters were grossly contaminated, with colony counts over $2{\cdot}5\times10^6$/cluster.

The milk spoilage organisms test, 3 days at 22°, was a most valuable addition to the colony count test. It was found to be a more sensitive indicator of the presence of milk spoilage organisms than the coli-aerogenes test and is recommended for routine use.

A study was made of the rinse results obtained with milking machine clusters cleansed by different methods. Results for milking machine clusters sterilized with steam were much better than those obtained for clusters claimed to have been cleansed by chemical methods.

The Effect of Freezing and Storage on the Flora of Pasteurized Milk. By J. G. Murray (*Ministry of Agriculture for Northern Ireland, and The Queen's University of Belfast*).

Milk pasteurized by the H.T.S.T. method was frozen at −28° in polythene bags. The effect of freezing and storage on the number of bacteria and on the flora of the pasteurized milk was studied, and also the effect of this treatment on contaminants likely to be found in milk, namely, some of the coli-aerogenes group. The colony count on the pasteurized and frozen milk was determined at 3°, 22°, 30° and 37°. Samples of frozen milk were examined immediately after freezing and replicate samples stored at −12° were examined at intervals up to 6 months.

The colony counts at 3° were consistently low on all samples and rarely exceeded 100/ml. The difference between the means at 30° and 37° of the pasteurized and frozen samples was not significant; at 22° it was highly significant. At all temperatures the difference between samples was very highly significant. The counts at 30° and 37° showed a highly significant interaction between treatment and sample. With the 22° count the interaction was not significant.

The effect of storage at −12° on the total count at 3°, 22°, 30° and 37° was determined on groups of samples daily for 5 days, and on a number of samples at intervals up to 6 months. The difference between counts at the early stages of storage was not significant. Differences between the samples were very highly significant. The interaction between storage treatment and sample was not significant. A significant decrease in the colony count at all temperatures coincided with a definite physical deterioration in the frozen product when melted, and this was always evident after 18 weeks storage at −12°. The samples inoculated with coli-aerogenes types also showed a highly significant drop in numbers at this stage.

The Growth of Bacteria in a Percolating Sewage Filter. By G. C. Ware and J. E. Loveless (*Department of Bacteriology, University of Bristol, Canynge Hall, Whatley Road, Bristol*, 8, *and The Water Pollution Research Station, Watford*). (See *J. appl. Bact.* **21**, 308).

Dilutions without Tears. By G. C. Ware (*Department of Bacteriology, University of Bristol, Canynge Hall, Whatley Road, Bristol*, 8).

From a stock solution of known strength a solution of any weaker strength may be prepared without calculation, if a volume of stock solution numerically equal to the concentration of the required dilution, measured in any units, is diluted to a total volume numerically equal to the concentration of the stock solution expressed in the same units. The resulting solution will be of the required concentration.

Example 1. Stock solution, 6·0%; strength required, 1·42%. Measure 1·42 ml of stock solution and dilute this to a total volume of 6·0 ml.

Example 2. Stock solution, 76 oz/m^3; strength required, 5 oz/m^3. Take 5 equal measures of the stock solution and dilute to a total of 76 equal measures.

Serial halving dilutions of commonly used concentrations are unwieldy. For instance, with a concentration of 100 iu/ml, halving dilutions in a series of 11 tubes will give 100, 50, 25, 12·5, 6·25, 3·125, 1·5625, 0·78125, 0·390625, 0·1953125 and 0·09765625 iu/ml.

Simplification is obtained by using starting concentrations which are numerically equal to an integral power of two, for halving dilutions, three for one third dilutions, and so on. Thus, starting with 1024 mg/100 ml, halving dilutions in an 11 tube series will give 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 mg/100 ml.

Again, from a solution containing 19,683 μg/ml, one third dilutions in a 10 tube series will give 19,683, 6,561, 2,187, 729, 243, 81, 27, 9, 3 and 1 μg/ml.

The Assay of Small Amounts of Organic Mercurials by Microbiological Methods. By G. Sykes (*Boots Pure Drug Co., Ltd., Microbiology Division, Nottingham*).

The agar plate diffusion and serial tube dilution techniques have been applied to the assay of mercurials when used as preservatives in pharmaceutical preparations. Chemical methods of assay at such low concentrations are unreliable. The plate diffusion technique follows that commonly used for the assay of antibiotics, the test organism being a selected *Staphylococcus aureus* strain; the tube dilution technique consists of preparing suitable serial dilutions in a nutrient medium, inoculating with the chosen culture (*Escherichia coli*, NCTC 5934) and reading the growth after only 4 hr incubation at 37°. On balance the plate method is the one of choice, although the tube method is the more sensitive. Suitable controls are necessary in both cases to counter the influence of interfering substances.

Phenylmercuric salts and thiomersal can be detected down to about 1 p/m by the diffusion method and to about 0·05 p/m by the tube dilution method.

The following papers were read on 10 July:—

Cold-Tolerant Fermentative Gram-negative Organisms from Meat and Other Sources. By B. P. Eddy and A. G. Kitchell (*Low Temperature Research Station, Downing Street, Cambridge*).

Twenty-eight strains of coli-aerogenes bacteria and one *Aeromonas* have been isolated which grow well at +1·5° and some at −1·5°. The optimum growth temperature for most of these strains was nearer 37° than 30°. Nine strains (including the *Aeromonas*) fermented lactose rapidly, the remainder slowly or not at all. All the rapid lactose fermenters gave a positive presumptive coli-aerogenes reaction in MacConkey's broth at 30°, but only 6 were positive at 37°; none was positive at 44°.

A Note on the Faecal Streptococci in Bovine Faeces. By T. F. Medrek (*Low Temperature Research Station, Downing Street, Cambridge*).

A quantitative and qualitative study has been made of the faecal streptococcal flora of bovine faeces. The geometric mean of the total streptococcal count was $1{\cdot}2\times10^5$. *Streptococcus bovis* was the predominant faecal streptococcus in 19 of the 21 samples examined. Unclassified and *Strep. faecium* strains were found in 8 of the samples. *Strep. faecalis* and its variants were absent, with the exception of a single isolate of *Strep. faecalis* var. *liquefaciens*.

The study indicates that the faecal streptococcal flora of bovine faeces is characterized by a significant predominance of raffinose-fermenting streptococci (91% of the isolates studied) which are not necessarily *Strep. bovis*. The relative absence of sorbitol- and tellurite-resistant strains is correspondingly striking.

The Mycological Formation of Fat in Agitated, Aerated Cultures. By T. K. Walker (*Department of Chemistry, The Manchester College of Science and Technology, Manchester*, 1). (See *J. appl. Bact.* **21**, 291).

The Hygienic Condition of Mis-used Milk Bottles after Washing. By J. G. Davis (*Dr. J. G. Davis & Partners*, 9, Gerrard Street, London, W.1).

Twelve experiments were made in 6 dairies, two in the London area, two in the Midlands and two in the North of England. Mis-used bottles were taken at random and put through the washer in the ordinary way; they were then examined by the Ministry of Agriculture rinse method. The rinse was also examined for the presence of presumptive coli-aerogenes bacteria and milk souring organisms. An approximately equal number of normal bottles was tested at the same time.

It was found that when the foreign matter was impervious, e.g. paint, cement, greasy smears, insects, etc., the rinse counts were of the same order as those of the normal bottle, and nearly always comfortably below the Ministry of Agriculture standard of 200/bottle. When, however, the foreign matter was pervious, e.g. chalk, soot, slime, moulds, etc., rinse counts appreciably higher than 200 were obtained, but presumptive coli-aerogenes bacteria could not be detected in the rinse. Only with bottles in very bad condition was it possible to detect the presence of milk souring organisms.

It is suggested, therefore, that although milk bottles retaining foreign matter after passing through a bottle washer are aesthetically objectionable they are unlikely to be a danger to public health.

Factors Controlling Sporulation of Yeasts. By R. R. Fowell and Margaret E. Moorse (*The Distillers Company Ltd., Research and Development Department, Great Burgh, Epsom, Surrey*).

Investigations have shown that yeasts tend to dissociate into a mixture of cell types with different powers of sporulation. Single-cell isolates of recent origin should therefore be used to study the conditions that govern sporulation. Isolation of cells from a yeast that has suffered a decline in sporulating ability provides a method of restoring, or even improving, its capacity to produce 4-spored asci.

The degree of sporulation exhibited by a yeast is markedly influenced by presporulation treatment. The presporulation phase is essentially an anaerobic growth stage, while the sporulation phase is aerobic and cell division is restricted. Sharp separation of the two phases is essential, not only for rapid formation of spores, but also for accurate assessment of the factors that operate in the two phases.

The superiority of liquid presporulation media, compared with solid media, has been confirmed for malt wort and tomato juice broth. Excess storage of fatty reserves was found to be detrimental for sporulation. The age of a fermenting malt wort culture influences sporulating ability, which declines rapidly when cells are harvested towards the end of fermentation; maximal sporulation is given by a culture after 85–90% of the CO_2 has been evolved.

The bakers' yeast used in these studies gave the same degree of sporulation on sodium acetate agar (Fowell, 1952) as on glucose-acetate-glycine agar (Kleyn, 1954) but maximal sporulation was attained more rapidly on Fowell's medium. Experiments have shown that other carbon substrates, such as glucose and sodium pyruvate, can effectively replace sodium acetate in agar media, provided their concentration

is limited and the pH is suitably adjusted. Thus 0·3% sodium pyruvate agar, pH 7·6, and 0·1% glucose agar, pH 7·6, gave as good results for sporulation as Fowell's medium.

References

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Kleyn, J. G. (1954). A study of some environmental factors controlling sporulation of yeast using a new sporulation medium. *Wallerstein Labs. Commun.* **17,** 91.

Relation Between Type of Soil and Errors Between and Within Dilutions when Making Soil Counts. By Mary Bateson (*National Agricultural Advisory Service, Shardlow Hall, Shardlow, Derby*).

Each of 10 sites was sampled on five occasions between October, 1956, and April, 1957. Two series of tenfold dilutions were prepared from the original suspension (50 g of soil in 500 ml of sterile quarter-strength Ringer's solution), one by changing pipettes at each dilution level ('change'), and the other using one pipette for the first dilution from the suspension and one pipette for all successive dilutions ('no change'). Triplicate plates were made from 10^{-4} to 10^{-12} dilutions on soil extract agar and incubated for 14 days at 25°.

Considering the 10^{-4}, 10^{-5} and 10^{-6} dilutions, the ratio of colony numbers at successive dilutions averaged about 1:6 for 'change' and 1:4 for 'no change'. Out of 134 sets of replicates at these dilution levels with results by both methods, excessive values of chi-squares were given by 60 'change' and 62 'no change' results. The distribution of values at $P=0{\cdot}01$ or greater was virtually the same.

Variability between samples from the same site appeared to be as important as differences between sites.

Autumn Meeting, 1958

For the Autumn Meeting of the Society, held on Tuesday, 21 October, 1958, visits were paid to the Laboratories and Works of Oxo, Ltd., Southwark Bridge Road, London, S.E.1, and Crosse & Blackwell, Ltd., Crimscott Street, London, S.E.1.

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